

## 《 1231 》 WATER FOR PHARMACEUTICAL PURPOSES

### INTRODUCTION

Water is widely used as a raw material, ingredient, and solvent in the processing, formulation, and manufacture of pharmaceutical products, active pharmaceutical ingredients (APIs) and intermediates, compendial articles, and analytical reagents. This general information chapter provides additional information about water, its quality attributes that are not included within a water monograph, processing techniques that can be used to improve water quality, and a description of minimum water quality standards that should be considered when selecting a water source.

This information chapter is not intended to replace existing regulations or guides that already exist to cover USA and International (ICH or WHO) GMP issues, engineering guides, or other regulatory (FDA, EPA, or WHO) guidances for water. The contents will help users to better understand pharmaceutical water issues and some of the microbiological and chemical concerns unique to water. This chapter is not an all-inclusive writing on pharmaceutical waters. It contains points that are basic information to be considered, when appropriate, for the processing, holding, and use of water. It is the user's responsibility to assure that pharmaceutical water and its production meet applicable governmental regulations, guidances, and the compendial specifications for the types of water used in compendial articles.

Control of the chemical purity of these waters is important and is the main purpose of the monographs in this compendium. Unlike other official articles, the bulk water monographs (*Purified Water* and *Water for Injection*) also limit how the article can be produced because of the belief that the nature and robustness of the purification process is directly related to the resulting purity. The chemical attributes listed in these monographs should be considered as a set of minimum specifications. More stringent specifications may be needed for some applications to ensure suitability for particular uses. Basic guidance on the appropriate applications of these waters is found in the monographs and is further explained in this chapter.

Control of the microbiological quality of water is important for many of its uses. Most packaged forms of water that have monograph standards are required to be sterile because some of their intended uses require this attribute for health and safety reasons. USP has determined that a microbial specification for the bulk monographed waters is inappropriate and has not been included within the monographs for these waters. These waters can be used in a variety of applications, some requiring extreme microbiological control and others requiring none. The needed microbial specification for a given bulk water depends upon its use. A single specification for this difficult-to-control attribute would unnecessarily burden some water users with irrelevant specifications and testing. However, some applications may require even more careful microbial control to avoid

the proliferation of microorganisms ubiquitous to water during the purification, storage, and distribution of this substance. A microbial specification would also be inappropriate when related to the “utility” or continuous supply nature of this raw material. Microbial specifications are typically assessed by test methods that take at least 48 to 72 hours to generate results. Because pharmaceutical waters are generally produced by continuous processes and used in products and manufacturing processes soon after generation, the water is likely to have been used well before definitive test results are available. Failure to meet a compendial specification would require investigating the impact and making a pass/fail decision on all product lots between the previous sampling's acceptable test result and a subsequent sampling's acceptable test result. The technical and logistical problems created by a delay in the result of such an analysis do not eliminate the user's need for microbial specifications. Therefore, such water systems need to be operated and maintained in a controlled manner that requires that the system be validated to provide assurance of operational stability and that its microbial attributes be quantitatively monitored against established alert and action levels that would provide an early indication of system control. The issues of water system validation and alert/action levels and specifications are included in this chapter.

### **SOURCE OR FEED WATER CONSIDERATIONS**

To ensure adherence to certain minimal chemical and microbiological quality standards, water used in the production of drug substances or as source or feed water for the preparation of the various types of purified waters must meet the requirements of the National Primary Drinking Water Regulations (NPDWR) (40 CFR 141) issued by the U.S. Environmental Protection Agency (EPA) or the drinking water regulations of the European Union or Japan, or the WHO drinking water guidelines. Limits on the types and quantities of certain organic and inorganic contaminants ensure that the water will contain only small, safe quantities of potentially objectionable chemical species. Therefore, water pretreatment systems will only be challenged to remove small quantities of these potentially difficult-to-remove chemicals. Also, control of objectionable chemical contaminants at the source-water stage eliminates the need to specifically test for some of them (e.g., trihalomethanes and heavy metals) after the water has been further purified.

Microbiological requirements of drinking water ensure the absence of coliforms, which, if determined to be of fecal origin, may indicate the potential presence of other potentially pathogenic microorganisms and viruses of fecal origin. Meeting these microbiological requirements does not rule out the presence of other microorganisms, which could be considered undesirable if found in a drug substance or formulated product.

To accomplish microbial control, Municipal Water Authorities add disinfectants to drinking water. Chlorine-containing and other oxidizing substances have been used for many

decades for this purpose and have generally been considered to be relatively innocuous to humans. However, these oxidants can interact with naturally occurring organic matter to produce disinfection by-products (DBPs), such as trihalomethanes (THMs, including chloroform, bromodichloromethane, and dibromochloromethane) and haloacetic acids (HAAs, including dichloroacetic acid and trichloroacetic acid). The levels of DBPs produced vary with the level and type of disinfectant used and the levels and types of organic materials found in the water, which can vary seasonally.

Because high levels of DBPs are considered a health hazard in drinking water, Drinking Water Regulations mandate their control to generally accepted nonhazardous levels. However, depending on the unit operations used for further water purification, a small fraction of the DBPs in the starting water may carry over to the finished water. Therefore, the importance of having minimal levels of DBPs in the starting water, while achieving effective disinfection, is important.

DBP levels in drinking water can be minimized by using disinfectants such as ozone, chloramines, or chlorine dioxide. Like chlorine, their oxidative properties are sufficient to damage some pretreatment unit operations and must be removed early in the pretreatment process. The complete removal of some of these disinfectants can be problematic. For example, chloramines may degrade during the disinfection process or during pretreatment removal, thereby releasing ammonia, which in turn can carry over to the finished water. Pretreatment unit operations must be designed and operated to adequately remove the disinfectant, drinking water DBPs, and objectionable disinfectant degradants. A serious problem can occur if unit operations designed to remove chlorine were, without warning, challenged with chloramine-containing drinking water from a municipality that had been mandated to cease use of chlorine disinfection to comply with ever tightening EPA Drinking Water THM specifications. The dechlorination process might incompletely remove the chloramine, which could irreparably damage downstream unit operations, but also the release of ammonia during this process might carry through pretreatment and prevent the finished water from passing compendial conductivity specifications. The purification process must be reassessed if the drinking water disinfectant is changed, emphasizing the need for a good working relationship between the pharmaceutical water manufacturer and the drinking water provider.

## TYPES OF WATER

There are many different grades of water used for pharmaceutical purposes. Several are described in *USP* monographs that specify uses, acceptable methods of preparation, and quality attributes. These waters can be divided into two general types: bulk waters, which are typically produced on site where they are used; and sterile waters, which are produced, packaged, and sterilized to preserve microbial quality throughout their packaged shelf life. There are several specialized types of sterile waters, differing in their

designated applications, packaging limitations, and other quality attributes.

There are also other types of water for which there are no monographs. These are all bulk waters, with names given for descriptive purposes only. Many of these waters are used in specific analytical methods. The associated text may not specify or imply certain quality attributes or modes of preparation. These nonmonographed waters may not necessarily adhere strictly to the stated or implied modes of preparation or attributes. Waters produced by other means or controlled by other test attributes may equally satisfy the intended uses for these waters. It is the user's responsibility to ensure that such waters, even if produced and controlled exactly as stated, be suitable for their intended use. Wherever the term “water” is used within this compendia without other descriptive adjectives or clauses, the intent is that water of no less purity than *Purified Water* be used.

What follows is a brief description of the various types of pharmaceutical waters and their significant uses or attributes. [Figure 1](#) may also be helpful in understanding some of the various types of waters.

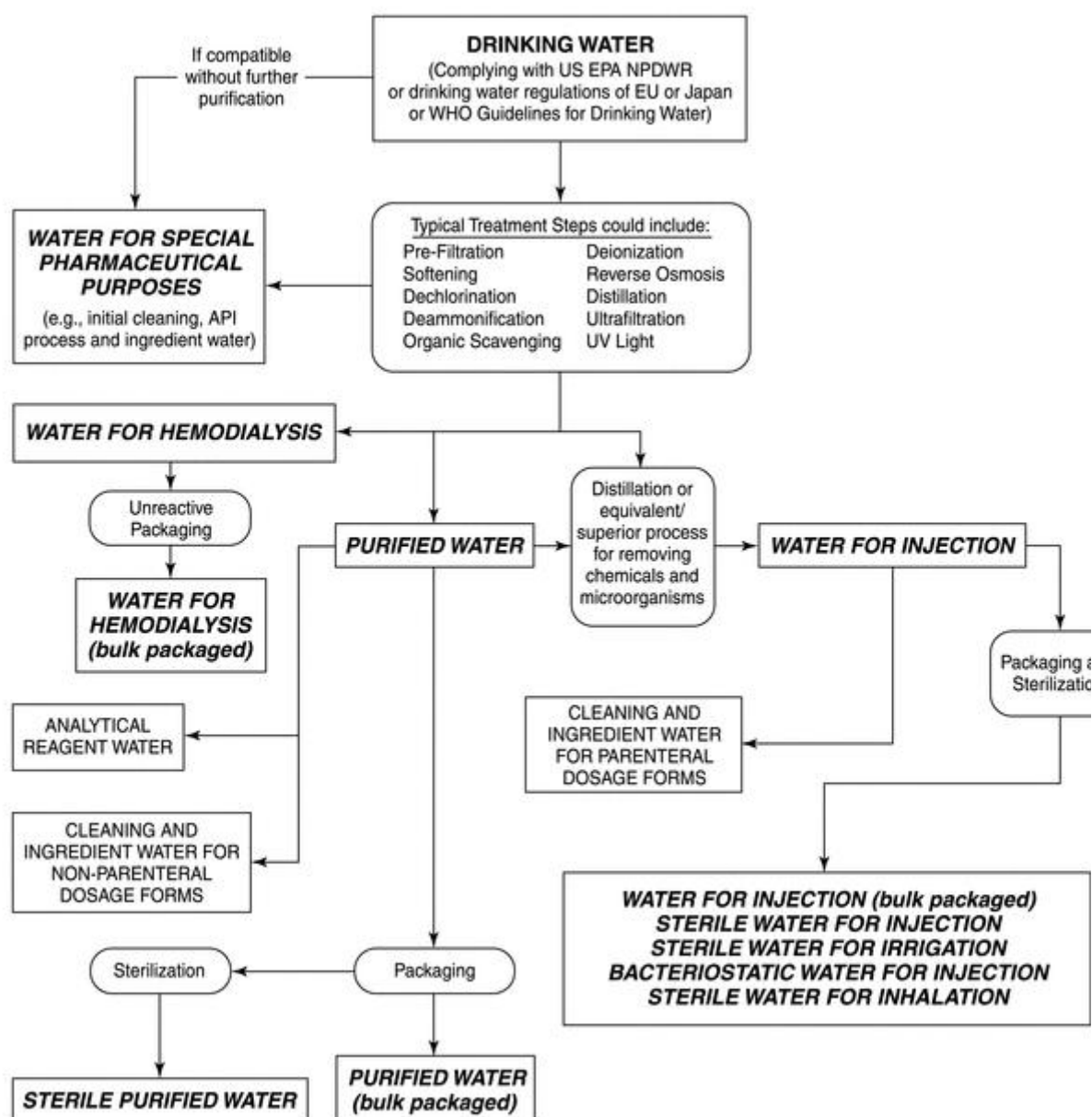


Figure 1. Water for pharmaceutical purposes.

### Bulk Monographed Waters and Steam

The following waters are typically produced in large volume by a multiple-unit operation water system and distributed by a piping system for use at the same site. These particular pharmaceutical waters must meet the quality attributes as specified in the related monographs.

**Purified Water**— [Purified Water](#) (see the *USP* monograph) is used as an excipient in the production of nonparenteral preparations and in other pharmaceutical applications, such as cleaning of certain equipment and nonparenteral product-contact components. Unless

otherwise specified, *Purified Water* is also to be used for all tests and assays for which water is indicated (see *General Notices and Requirements*). *Purified Water* is also referenced throughout the *USP – NF*. Regardless of the font and letter case used in its spelling, water complying with the *Purified Water* monograph is intended. *Purified Water* must meet the requirements for ionic and organic chemical purity and must be protected from microbial contamination. The minimal quality of source or feed water for the production of *Purified Water* is Drinking Water. This source water may be purified using unit operations that include deionization, distillation, ion exchange, reverse osmosis, filtration, or other suitable purification procedures. Purified water systems must be validated to reliably and consistently produce and distribute water of acceptable chemical and microbiological quality. Purified water systems that function under ambient conditions are particularly susceptible to the establishment of tenacious biofilms of microorganisms, which can be the source of undesirable levels of viable microorganisms or endotoxins in the effluent water. These systems require frequent sanitization and microbiological monitoring to ensure water of appropriate microbiological quality at the points of use. The *Purified Water* monograph also allows bulk packaging for commercial use elsewhere. There is a potential for the occurrence of microbial contamination and other quality changes in this bulk packaged nonsterile water. Therefore, this form of *Purified Water* should be prepared and stored in a fashion that limits microbial growth and/or simply used in a timely fashion before microbial proliferation renders it unsuitable for its intended use. Also depending on the material used for packaging, there could be extractable compounds leaching into the water from the packaging. Though this article may meet its required chemical attributes, such extractables may render the water an inappropriate choice for some applications. It is the user's responsibility to ensure fitness for use of this packaged article when used in manufacturing, clinical, or analytical applications where the pure bulk form of the water is indicated.

**Water for Injection**— [Water for Injection](#) (see the *USP* monograph) is used as an excipient in the production of parenteral and other preparations where product endotoxin content must be controlled, and in other pharmaceutical applications, such as cleaning of certain equipment and parenteral product-contact components. The minimum quality of source or feed water for the generation of [Water for Injection](#) is Drinking Water as defined by the U.S. EPA, EU, Japan, or WHO. This source water may be pretreated to render it suitable for subsequent distillation (or whatever other validated process is used according to the monograph). The finished water must meet all of the chemical requirements for *Purified Water* as well as an additional bacterial endotoxin specification. Since endotoxins are produced by the kinds of microorganisms that are prone to inhabit water, the equipment and procedures used by the system to purify, store, and distribute *Water for Injection* must be designed to minimize or prevent microbial contamination as well as remove incoming endotoxins from the starting water. *Water for Injection* systems must be

validated to reliably and consistently produce and distribute this quality of water.

The *Water for Injection* monograph also allows it to be packed in bulk for commercial use.

Bulk packaged *Water for Injection* is required to be sterile, thus eliminating microbial contamination quality changes. However, packaging extractables may render this water an inappropriate choice for some applications. It is the user's responsibility to ensure fitness for use of this packaged article when used in manufacturing, clinical, or analytical applications where the purer bulk form of the water is indicated.

**Water for Hemodialysis**— [Water for Hemodialysis](#) (see the *USP* monograph) is used for hemodialysis applications, primarily the dilution of hemodialysis concentrate solutions. It is produced and used on site and is made from EPA Drinking Water which has been further purified to reduce chemical and microbiological components. It may be packaged and stored in unreactive containers that preclude bacterial entry. The term “unreactive containers” implies that the container, especially its water contact surfaces, are not changed in any way by the water, such as by leaching of container-related compounds into the water or by any chemical reaction or corrosion caused by the water. The water contains no added antimicrobials and is not intended for injection. Its attributes include specifications for *Water conductivity*, *Total organic carbon* (or oxidizable substances), *Microbial limits*, and *Bacterial endotoxins*. The water conductivity and total organic carbon attributes are identical to those established for *Purified Water* and *Water for Injection*; however, instead of total organic carbon, the organic content may alternatively be measured by the test for *Oxidizable substances*. The *Microbial limits* attribute for this water is unique among the “bulk” water monographs, but is justified on the basis of this water's specific application that has microbial content requirements related to its safe use. The *Bacterial endotoxins* attribute is likewise established at a level related to its safe use.

**Pure Steam**— [Pure Steam](#) (see the *USP* monograph) is also sometimes referred to as “clean steam” . It is used where the steam or its condensate would directly contact official articles or article-contact surfaces, such as during their preparation, sterilization, or cleaning where no subsequent processing step is used to remove any codeposited impurity residues. These *Pure Steam* applications include but are not limited to porous load sterilization processes, product or cleaning solutions heated by direct steam injection, or humidification of processes where steam injection is used to control the humidity inside processing vessels where the official articles or their in-process forms are exposed. The primary intent of using this quality of steam is to ensure that official articles or article-contact surfaces exposed to it are not contaminated by residues within the steam.

*Pure Steam* is prepared from suitably pretreated source water analogously to either the pretreatment used for *Purified Water* or *Water for Injection*. The water is vaporized with suitable mist elimination, and distributed under pressure. The sources of undesirable contaminants within *Pure Steam* could arise from entrained source water droplets,

anticorrosion steam additives, or residues from the steam production and distribution system itself. The attributes in the [Pure Steam](#) monograph should detect most of the contaminants that could arise from these sources. If the official article exposed to potential *Pure Steam* residues is intended for parenteral use or other applications where the pyrogenic content must be controlled, the *Pure Steam* must additionally meet the specification for [Bacterial Endotoxins](#) [〈 85 〉](#).

These purity attributes are measured on the condensate of the article, rather than the article itself. This, of course, imparts great importance to the cleanliness of the *Pure Steam* condensate generation and collection process because it must not adversely impact the quality of the resulting condensed fluid.

Other steam attributes not detailed in the monograph, in particular, the presence of even small quantities of noncondensable gases or the existence of a superheated or dry state, may also be important for applications such as sterilization. The large release of energy (latent heat of condensation) as water changes from the gaseous to the liquid state is the key to steam's sterilization efficacy and its efficiency, in general, as a heat transfer agent. If this phase change (condensation) is not allowed to happen because the steam is extremely hot and in a persistent superheated, dry state, then its usefulness could be seriously compromised. Noncondensable gases in steam tend to stratify or collect in certain areas of a steam sterilization chamber or its load. These surfaces would thereby be at least partially insulated from the steam condensation phenomenon, preventing them from experiencing the full energy of the sterilizing conditions. Therefore, control of these kinds of steam attributes, in addition to its chemical purity, may also be important for certain *Pure Steam* applications. However, because these additional attributes are use-specific, they are not mentioned in the *Pure Steam* monograph.

Note that less pure “plant steam” may be used for steam sterilization of nonproduct contact nonporous loads, for general cleaning of nonproduct contact equipment, as a nonproduct contact heat exchange medium, and in all compatible applications involved in bulk pharmaceutical chemical and API manufacture.

### **Sterile Monographed Waters**

The following monographed waters are packaged forms of either *Purified Water* or *Water for Injection* that have been sterilized to preserve their microbiological properties. These waters may have specific intended uses as indicated by their names and may also have restrictions on packaging configurations related to those uses. In general, these waters may be used in lieu of the bulk form of water from which they were derived. However, the user should take into consideration that the packaging and sterilization processes used for the articles may leach materials from the packaging material into the water over its shelf life, rendering it less pure than the original water placed into the package. It is the user's responsibility to ensure fitness for use of this article when used in manufacturing, clinical, or analytical applications where the purer bulk form of the water



is indicated.

**Sterile Purified Water**— [Sterile Purified Water](#) (see the *USP* monograph) is *Purified Water*, packaged and rendered sterile. It is used in the preparation of nonparenteral compendial dosage forms or in analytical applications requiring *Purified Water* where access to a validated *Purified Water* system is not practical, where only a relatively small quantity is needed, where *Sterile Purified Water* is required, or where bulk packaged *Purified Water* is not suitably microbiologically controlled.

**Sterile Water for Injection**— [Sterile Water for Injection](#) (see the *USP* monograph) is *Water for Injection* packaged and rendered sterile. It is used for extemporaneous prescription compounding and as a sterile diluent for parenteral products. It may also be used for other applications where bulk *Water for Injection* or *Purified Water* is indicated but where access to a validated water system is either not practical or where only a relatively small quantity is needed. *Sterile Water for Injection* is packaged in single-dose containers not larger than 1 L in size.

**Bacteriostatic Water for Injection**— [Bacteriostatic Water for Injection](#) (see the *USP* monograph) is sterile *Water for Injection* to which has been added one or more suitable antimicrobial preservatives. It is intended to be used as a diluent in the preparation of parenteral products, most typically for multi-dose products that require repeated content withdrawals. It may be packaged in single-dose or multiple-dose containers not larger than 30 mL.

**Sterile Water for Irrigation**— [Sterile Water for Irrigation](#) (see the *USP* monograph) is *Water for Injection* packaged and sterilized in single-dose containers of larger than 1 L in size that allows rapid delivery of its contents. It need not meet the requirement under small-volume injections in the general test chapter [Particulate Matter in Injections](#) { 788 }. It may also be used in other applications which do not have particulate matter specifications, where bulk *Water for Injection* or *Purified Water* is indicated but where access to a validated water system is not practical, or where somewhat larger quantities than are provided as *Sterile Water for Injection* are needed.

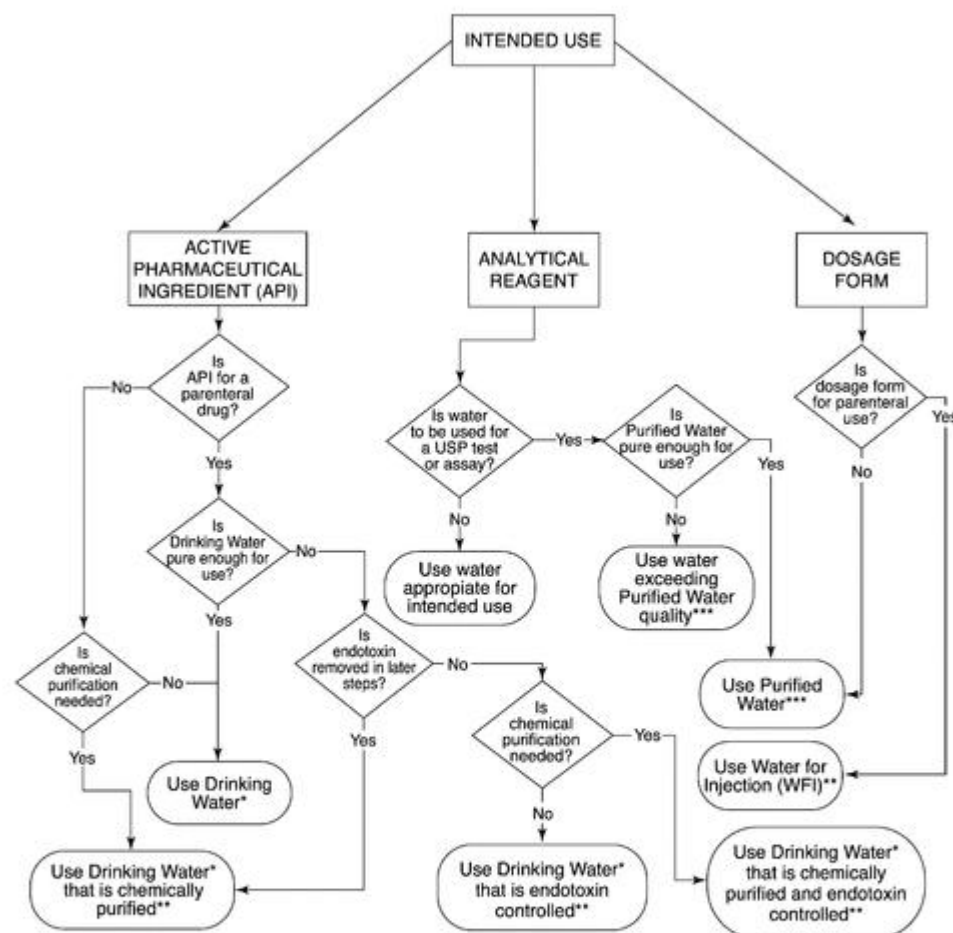
**Sterile Water for Inhalation**— [Sterile Water for Inhalation](#) (see the *USP* monograph) is *Water for Injection* that is packaged and rendered sterile and is intended for use in inhalators and in the preparation of inhalation solutions. It carries a less stringent specification for bacterial endotoxins than *Sterile Water for Injection* and therefore is not suitable for parenteral applications.

### Nonmonographed Manufacturing Waters

In addition to the bulk monographed waters described above, nonmonographed waters can also be used in pharmaceutical processing steps such as cleaning, synthetic steps, or a starting material for further purification. The following is a description of several of

these nonmonographed waters as cited in various locations within this compendia.

**Drinking Water**— This type of water can be referred to as Potable Water (meaning drinkable or fit to drink), National Primary Drinking Water, Primary Drinking Water, or National Drinking Water. Except where a singular drinking water specification is stated (such as the NPDWR [U.S. Environmental Protection Agency's National Primary Drinking Water Regulations as cited in 40 CFR Part 141]), this water must comply with the quality attributes of either the NPDWR, or the drinking water regulations of the European Union or Japan, or the WHO Drinking Water Guidelines. It may be derived from a variety of sources including a public water utility, a private water supply (e.g., a well), or a combination of these sources. Drinking Water may be used in the early stages of cleaning pharmaceutical manufacturing equipment and product-contact components. Drinking Water is also the minimum quality of water that should be used for the preparation of official substances and other bulk pharmaceutical ingredients. Where compatible with the processes, the allowed contaminant levels in Drinking Water are generally considered safe for use for official substances and other drug substances. Where required by the processing of the materials to achieve their required final purity, higher qualities of water may be needed for these manufacturing steps, perhaps even as pure as *Water for Injection* or *Purified Water*. Such higher purity waters, however, might require only selected attributes to be of higher purity than Drinking Water (see [Figure 2](#)). Drinking Water is the prescribed source or feed water for the production of bulk monographed pharmaceutical waters. The use of Drinking Water specifications establishes a reasonable set of maximum allowable levels of chemical and microbiological contaminants with which a water purification system will be challenged. As seasonal variations in the quality attributes of the Drinking Water supply can occur, due consideration to its synthetic and cleaning uses must be given. The processing steps in the production of pharmaceutical waters must be designed to accommodate this variability.



\* Drinking Water is water complying with US EPA NPDR or drinking water regulations of EU or Japan or WHO drinking water guidelines.

\*\* Water for sterile API's or dosage forms must first be rendered sterile if there is not a subsequent sterilization step in the process where used.

\*\*\* See guidance in this chapter where waters other than Purified Water are required by some USP tests and assays.

Note: All water systems should be validated with whatever microbial control is needed to suit the intended purposes of the water.

Figure 2. Selection of water for pharmaceutical purposes.

**Hot Purified Water**— This water is used in the preparation instructions for *USP – NF* articles and is clearly intended to be *Purified Water* that has been heated to an unspecified temperature in order to enhance solubilization of other ingredients. There is no upper temperature limit for the water (other than being less than 100°), but for each monograph there is an implied lower limit below which the desired solubilization effect would not occur.

### Nonmonographed Analytical Waters

Both *General Notices and Requirements* and the introductory section to *Reagents, Indicators, and Solutions* clearly state that where the term “water,” without qualification or other specification, is indicated for use in analyses, the quality of water shall be *Purified Water*. However, numerous such qualifications do exist. Some of these qualifications involve methods of preparation, ranging from specifying the primary

purification step to specifying additional purification. Other qualifications call for specific attributes to be met that might otherwise interfere with analytical processes. In most of these latter cases, the required attribute is not specifically tested. Rather, a further “purification process” is specified that ostensibly allows the water to adequately meet this required attribute.

However, preparation instructions for many reagents were carried forward from the innovator's laboratories to the originally introduced monograph for a particular *USP – NF* article or general test chapter. The quality of the reagent water described in these tests may reflect the water quality designation of the innovator's laboratory. These specific water designations may have originated without the innovator's awareness of the requirement for *Purified Water* in *USP – NF* tests. Regardless of the original reason for the creation of these numerous special analytical waters, it is possible that the attributes of these special waters could now be met by the basic preparation steps and current specifications of *Purified Water*. In some cases, however, some of the cited post-processing steps are still necessary to reliably achieve the required attributes.

Users are not obligated to employ specific and perhaps archaically generated forms of analytical water where alternatives with equal or better quality, availability, or analytical performance may exist. The consistency and reliability for producing these alternative analytical waters should be verified as producing the desired attributes. In addition, any alternative analytical water must be evaluated on an application-by-application basis by the user to ensure its suitability. Following is a summary of the various types of nonmonographed analytical waters that are cited in the *USP – NF*.

**Distilled Water**— This water is produced by vaporizing liquid water and condensing it in a purer state. It is used primarily as a solvent for reagent preparation, but it is also specified in the execution of other aspects of tests, such as for rinsing an analyte, transferring a test material as a slurry, as a calibration standard or analytical blank, and for test apparatus cleaning. It is also cited as the starting water to be used for making *High Purity Water*. Because none of the cited uses of this water imply a need for a particular purity attribute that can only be derived by distillation, water meeting the requirements for *Purified Water* derived by other means of purification could be equally suitable where *Distilled Water* is specified.

**Freshly Distilled Water**— Also called “recently distilled water”, it is produced in a similar fashion to *Distilled Water* and should be used shortly after its generation. This implies the need to avoid endotoxin contamination as well as any other adventitious forms of contamination from the air or containers that could arise with prolonged storage. It is used for preparing solutions for subcutaneous test animal injections as well as for a reagent solvent in tests for which there appears to be no particularly high water purity needed that could be ascribable to being “freshly distilled”. In the “test-animal” use, the term “freshly distilled” and its testing use imply a chemical, endotoxin, and

microbiological purity that could be equally satisfied by *Water for Injection* (though no reference is made to these chemical, endotoxin, or microbial attributes or specific protection from recontamination). For nonanimal uses, water meeting the requirements for *Purified Water* derived by other means of purification and/or storage periods could be equally suitable where “recently distilled water” or *Freshly Distilled Water* is specified.

**Deionized Water**— This water is produced by an ion-exchange process in which the contaminating ions are replaced with either  $H^+$  or  $OH^-$  ions. Similarly to *Distilled Water*, *Deionized Water* is used primarily as a solvent for reagent preparation, but it is also specified in the execution of other aspects of tests, such as for transferring an analyte within a test procedure, as a calibration standard or analytical blank, and for test apparatus cleaning. Also, none of the cited uses of this water imply any needed purity attribute that can only be achieved by deionization. Therefore, water meeting the requirements for *Purified Water* that is derived by other means of purification could be equally suitable where *Deionized Water* is specified.

**Freshly Deionized Water**— This water is prepared in a similar fashion to *Deionized Water*, though as the name suggests, it is to be used shortly after its production. This implies the need to avoid any adventitious contamination that could occur upon storage. This water is indicated for use as a reagent solvent as well as for cleaning. Due to the nature of the testing, *Purified Water* could be a reasonable alternative for these applications.

**Deionized Distilled Water**— This water is produced by deionizing (see *Deionized Water*) *Distilled Water*. This water is used as a reagent in a liquid chromatography test that requires a high purity. Because of the importance of this high purity, water that barely meets the requirements for *Purified Water* may not be acceptable. *High Purity Water* (see below) could be a reasonable alternative for this water.

**Filtered Distilled or Deionized Water**— This water is essentially *Purified Water* produced by distillation or deionization that has been filtered through a 1.2- $\mu m$  rated membrane. This water is used in particulate matter testing where the presence of particles in the water could bias the test results (see [Particulate Matter in Injections](#) § 788). Because the chemical water purity needed for this test could also be afforded by water purification processes other than distillation or deionization, filtered water meeting the requirements for *Purified Water* but produced by means other than distillation or deionization could be equally suitable.

**Filtered Water**— This water is *Purified Water* that has been filtered to remove particles that could interfere with the analysis where the water is used. Where used for preparing samples for particulate matter testing (see [Particulate Matter in Injections](#) § 788), though unspecified in monographs, water filtration should be through a 1.2- $\mu m$  filter to be consistent with the general test chapter. Where used as a chromatography reagent,

monograph-specified filter ratings range from 0.5 µm to unspecified.

**High Purity Water**— The preparation of this water is defined in [Containers—Glass](#) ( 660 ). It is water that is prepared by deionizing previously distilled water, and then filtering it through a 0.45-µm rated membrane. This water must have an in-line conductivity of not greater than 0.15 µS/cm (6.67 Megohm-cm) at 25°. For the sake of purity comparison, the analogous Stage 1 and 2 conductivity requirements for *Purified Water* at the same temperature are 1.3 µS/cm and 2.1 µS/cm, respectively. The preparation specified in [Containers—Glass](#) ( 660 ) uses materials that are highly efficient deionizers and that do not contribute copper ions or organics to the water, assuring a very high quality water. If the water of this purity contacts the atmosphere even briefly as it is being used or drawn from its purification system, its conductivity will immediately degrade, by as much as about 1.0 µS/cm, as atmospheric carbon dioxide dissolves in the water and equilibrates to bicarbonate ions. Therefore, if the analytical use requires that water purity remains as high as possible, its use should be protected from atmospheric exposure. This water is used as a reagent, as a solvent for reagent preparation, and for test apparatus cleaning where less pure waters would not perform acceptably. However, if a user's routinely available purified water is filtered and meets or exceeds the conductivity specifications of *High Purity Water*, it could be used in lieu of *High Purity Water*.

**Ammonia-Free Water**— Functionally, this water must have a negligible ammonia concentration to avoid interference in tests sensitive to ammonia. It has been equated with *High Purity Water* that has a significantly tighter Stage 1 conductivity specification than *Purified Water* because of the latter's allowance for a minimal level of ammonium among other ions. However, if the user's *Purified Water* were filtered and met or exceeded the conductivity specifications of *High Purity Water*, it would contain negligible ammonia or other ions and could be used in lieu of *High Purity Water*.

**Carbon Dioxide-Free Water**— The introductory portion of the *Reagents, Indicators, and Solutions* section defines this water as *Purified Water* that has been vigorously boiled for at least 5 minutes, then cooled and protected from absorption of atmospheric carbon dioxide. Because the absorption of carbon dioxide tends to drive down the water pH, most of the uses of *Carbon Dioxide-Free Water* are either associated as a solvent in pH-related or pH-sensitive determinations or as a solvent in carbonate-sensitive reagents or determinations. Another use of this water is for certain optical rotation and color and clarity of solution tests. Though it is possible that this water is indicated for these tests simply because of its purity, it is also possible that the pH effects of carbon dioxide containing water could interfere with the results of these tests. A third plausible reason that this water is indicated is that outgassing air bubbles might interfere with these photometric-type tests. The boiled water preparation approach will also greatly reduce the concentrations of many other dissolved gases along with carbon dioxide. Therefore, in some of the applications for *Carbon Dioxide-Free Water*, it could be the inadvertent

deaeration effect that actually renders this water suitable. In addition to boiling, deionization is perhaps an even more efficient process for removing dissolved carbon dioxide (by drawing the dissolved gas equilibrium toward the ionized state with subsequent removal by the ion-exchange resins). If the starting *Purified Water* is prepared by an efficient deionization process and protected after deionization from exposure to atmospheric air, water that is carbon dioxide-free can be effectively made without the application of heat. However this deionization process does not deaerate the water, so if *Purified Water* prepared by deionization is considered as a substitute water in a test requiring *Carbon Dioxide-Free Water*, the user must verify that it is not actually water akin to *Deaerated Water* (discussed below) that is needed for the test. As indicated in *High Purity Water*, even brief contact with the atmosphere can allow small amounts of carbon dioxide to dissolve, ionize, and significantly degrade the conductivity and lower the pH. If the analytical use requires the water to remain as pH-neutral and as carbon dioxide-free as possible, even the analysis should be protected from atmospheric exposure. However, in most applications, atmospheric exposure during testing does not significantly affect its suitability in the test.

**Ammonia- and Carbon Dioxide-Free Water**— As implied by the name, this water should be prepared by approaches compatible with those mentioned for both *Ammonia-Free Water* and *Carbon Dioxide-Free Water*. Because the carbon dioxide-free attribute requires post-production protection from the atmosphere, it is appropriate to first render the water ammonia-free using the *High Purity Water* process followed by the boiling and carbon dioxide-protected cooling process. The *High Purity Water* deionization process for creating *Ammonia-Free Water* will also remove the ions generated from dissolved carbon dioxide and ultimately, by forced equilibration to the ionized state, all the dissolved carbon dioxide. Therefore, depending on its use, an acceptable procedure for making *Ammonia- and Carbon Dioxide-Free Water* could be to transfer and collect *High Purity Water* in a carbon dioxide intrusion-protected container.

**Deaerated Water**— This water is *Purified Water* that has been treated to reduce the content of dissolved air by “suitable means” . In the *Reagents* section, approaches for boiling, cooling (similar to *Carbon Dioxide-Free Water* but without the atmospheric carbon dioxide protection), and sonication are given as applicable for test uses other than dissolution and drug release testing. Though *Deaerated Water* is not mentioned by name in [Dissolution](#) (711), suggested methods for deaerating dissolution media (which may be water) include warming to 41 °, vacuum filtering through a 0.45-µm rated membrane, and vigorously stirring the filtrate while maintaining the vacuum. This chapter specifically indicates that other validated approaches may be used. In other monographs that also do not mention *Deaerated Water* by name, degassing of water and other reagents is accomplished by sparging with helium. *Deaerated Water* is used in both dissolution testing as well as liquid chromatography applications where outgassing could either



interfere with the analysis itself or cause erroneous results due to inaccurate volumetric withdrawals. Applications where ambient temperature water is used for reagent preparation, but the tests are performed at elevated temperatures, are candidates for outgassing effects. If outgassing could interfere with test performance, including chromatographic flow, colorimetric or photometric measurements, or volumetric accuracy, then *Deaerated Water* should probably be used, whether called for in the analysis or not. The above deaeration approaches might not render the water “gas-free”. At best, they reduce the dissolved gas concentrations so that outgassing caused by temperature changes is not likely.

**Recently Boiled Water**— This water may include recently or freshly boiled water (with or without mention of cooling in the title), but cooling prior to use is clearly intended. Occasionally it is necessary to use when hot. *Recently Boiled Water* is specified because it is used in a pH-related test or carbonate-sensitive reagent, in an oxygen-sensitive test or reagent, or in a test where outgassing could interfere with the analysis, such as specific gravity or an appearance test.

**Oxygen-Free Water**— The preparation of this water is not specifically described in the compendia. Neither is there an oxygen specification or analysis mentioned. However, all uses involve analyses of materials that could be sensitive to oxidation by atmospheric oxygen. Procedures for the removal of dissolved oxygen from solvents, though not necessarily water, are mentioned in [Polarography](#) { 801 } and [Spectrophotometry and Light-Scattering](#) { 851 }. These procedures involve simple sparging of the liquid with an inert gas such as nitrogen or helium followed by inert gas blanketing to prevent oxygen reabsorption. The sparging times cited range from 5 to 15 minutes to an unspecified period. Some *Purified Water* and *Water for Injection* systems produce water that is maintained in a hot state and that is inert gas blanketed during its preparation and storage and distribution. Though oxygen is poorly soluble in hot water, such water may not be oxygen-free. Whatever procedure used for removing oxygen should be verified as reliably producing water that is fit for use.

**LAL Reagent Water**— This water is also referred to as endotoxin-free water. This is usually *Water for Injection*, which may have been sterilized. It is free from a level of endotoxin that would yield any detectable reaction or interference with the Limulus amoebocyte lysate reagent used in the [Bacterial Endotoxins Test](#) { 85 }.

**Organic-Free Water**— This water is defined by *Residual Solvents* { 467 } as producing no significantly interfering gas chromatography peaks. Referenced monographs specify using this water as the solvent for the preparation of standard and test solutions for the *Residual solvents* test.

**Lead-Free Water**— This water is used as a transferring diluent for an analyte in a [Lead](#) { 251 } test. Though no specific instructions are given for its preparation, it must not



contain any detectable lead. *Purified Water* should be a suitable substitute for this water.

**Chloride-Free Water**— This water is specified as the solvent for use in an assay that contains a reactant that precipitates in the presence of chloride. Though no specific preparation instructions are given for this water, its rather obvious attribute is having a very low chloride level in order to be unreactive with this chloride sensitive reactant. *Purified Water* could be used for this water but should be tested to ensure that it is unreactive.

**Hot Water**— The uses of this water include solvents for achieving or enhancing reagent solubilization, restoring the original volume of boiled or hot solutions, rinsing insoluble analytes free of hot water soluble impurities, solvents for reagent recrystallization, apparatus cleaning, and as a solubility attribute for various *USP - NF* articles. In only one monograph is the temperature of “hot” water specified; so in all the other cases, the water temperature is less important, but should be high enough to achieve the desirable effect. In all cases, the chemical quality of the water is implied to be that of *Purified Water*.

## VALIDATION AND QUALIFICATION OF WATER PURIFICATION, STORAGE, AND DISTRIBUTION SYSTEMS

Establishing the dependability of pharmaceutical water purification, storage, and distribution systems requires an appropriate period of monitoring and observation. Ordinarily, few problems are encountered in maintaining the chemical purity of *Purified Water* and *Water for Injection*. Nevertheless, the advent of using conductivity and TOC to define chemical purity has allowed the user to more quantitatively assess the water's chemical purity and its variability as a function of routine pretreatment system maintenance and regeneration. Even the presence of such unit operations as heat exchangers and use point hoses can compromise the chemical quality of water within and delivered from an otherwise well-controlled water system. Therefore, an assessment of the consistency of the water's chemical purity over time must be part of the validation program. However, even with the most well controlled chemical quality, it is often more difficult to consistently meet established microbiological quality criteria owing to phenomena occurring during and after chemical purification. A typical program involves intensive daily sampling and testing of major process points for at least one month after operational criteria have been established for each unit operation, point of use, and sampling point.

An overlooked aspect of water system validation is the delivery of the water to its actual location of use. If this transfer process from the distribution system outlets to the water use locations (usually with hoses) is defined as outside the water system, then this transfer process still needs to be validated to not adversely affect the quality of the water to the extent it becomes unfit for use. Because routine microbial monitoring is performed

for the same transfer process and components (e.g., hoses and heat exchangers) as that of routine water use (see *Sampling Considerations*), there is some logic to include this water transfer process within the distribution system validation.

Validation is the process whereby substantiation to a high level of assurance that a specific process will consistently produce a product conforming to an established set of quality attributes is acquired and documented. Prior to and during the very early stages of validation, the critical process parameters and their operating ranges are established. A validation program qualifies and documents the design, installation, operation, and performance of equipment. It begins when the system is defined and moves through several stages: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). A graphical representation of a typical water system validation life cycle is shown in [Figure 3](#).

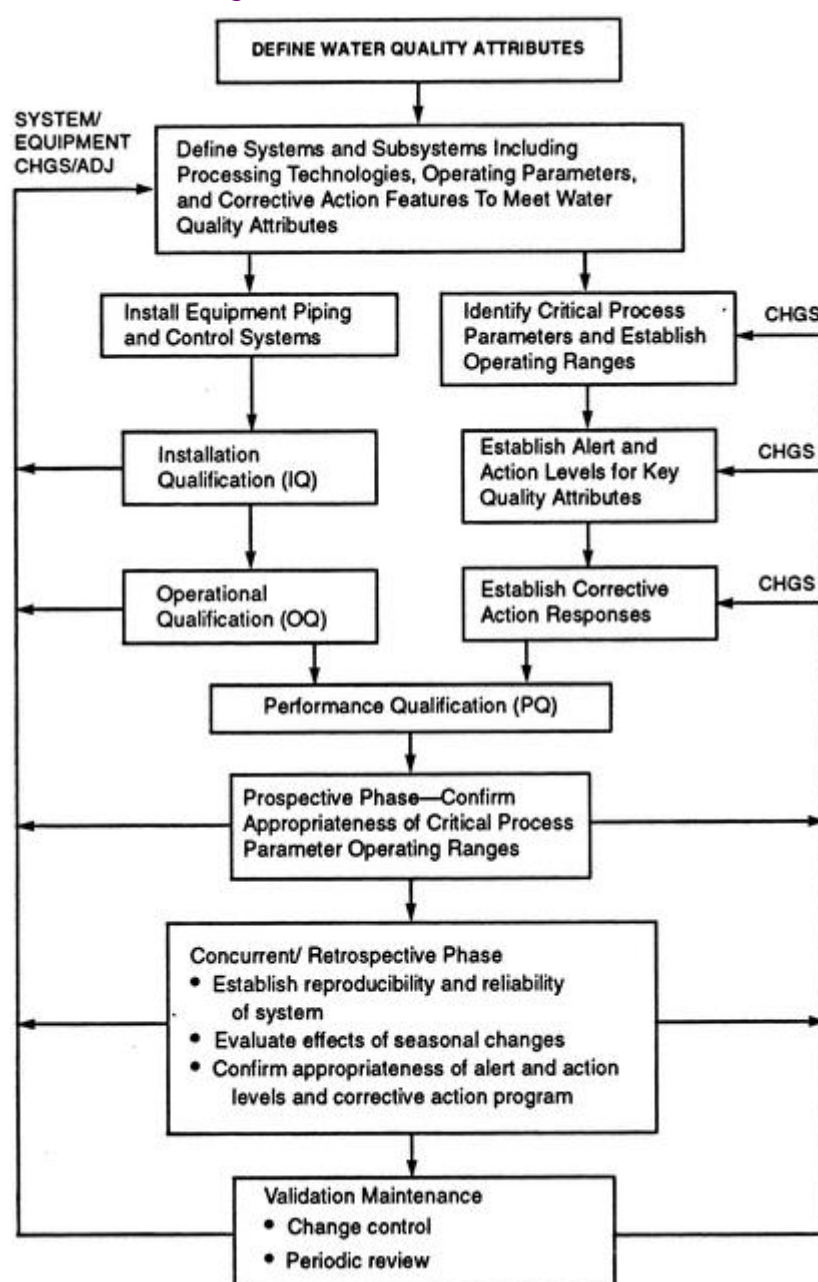


Fig. 3. Water system validation life cycle.

A validation plan for a water system typically includes the following steps: (1) establishing standards for quality attributes of the finished water and the source water; (2) defining suitable unit operations and their operating parameters for achieving the desired finished water quality attributes from the available source water; (3) selecting piping, equipment, controls, and monitoring technologies; (4) developing an IQ stage consisting of instrument calibrations, inspections to verify that the drawings accurately depict the final configuration of the water system and, where necessary, special tests to verify that the installation meets the design requirements; (5) developing an OQ stage consisting of tests and inspections to verify that the equipment, system alerts, and controls are operating reliably and that appropriate alert and action levels are established (This phase of qualification may overlap with aspects of the next step.); and (6) developing a prospective PQ stage to confirm the appropriateness of critical process parameter operating ranges (During this phase of validation, alert and action levels for key quality attributes and operating parameters are verified.); (7) assuring the adequacy of ongoing control procedures, e.g., sanitization frequency; (8) supplementing a validation maintenance program (also called continuous validation life cycle) that includes a mechanism to control changes to the water system and establishes and carries out scheduled preventive maintenance including recalibration of instruments (In addition, validation maintenance includes a monitoring program for critical process parameters and a corrective action program.); (9) instituting a schedule for periodic review of the system performance and requalification, and (10) completing protocols and documenting Steps 1 through 9.

## PURIFIED WATER AND WATER FOR INJECTION SYSTEMS

The design, installation, and operation of systems to produce *Purified Water* and *Water for Injection* include similar components, control techniques, and procedures. The quality attributes of both waters differ only in the presence of a bacterial endotoxin requirement for *Water for Injection* and in their methods of preparation, at least at the last stage of preparation. The similarities in the quality attributes provide considerable common ground in the design of water systems to meet either requirement. The critical difference is the degree of control of the system and the final purification steps needed to ensure bacterial and bacterial endotoxin removal.

Production of pharmaceutical water employs sequential unit operations (processing steps) that address specific water quality attributes and protect the operation of subsequent treatment steps. A typical evaluation process to select an appropriate water quality for a particular pharmaceutical purpose is shown in the decision tree in [Figure 2](#). This diagram may be used to assist in defining requirements for specific water uses and in the selection of unit operations. The final unit operation used to produce *Water for Injection* is limited to distillation or other processes equivalent or superior to distillation in the

removal of chemical impurities as well as microorganisms and their components.

Distillation has a long history of reliable performance and can be validated as a unit operation for the production of *Water for Injection*, but other technologies or combinations of technologies can be validated as being equivalently effective. Other technologies, such as ultrafiltration following other chemical purification process, may be suitable in the production of *Water for Injection* if they can be shown through validation to be as effective and reliable as distillation. The advent of new materials for older technologies, such as reverse osmosis and ultrafiltration, that allow intermittent or continuous operation at elevated, microbial temperatures, show promise for a valid use in producing *Water for Injection*.

The validation plan should be designed to establish the suitability of the system and to provide a thorough understanding of the purification mechanism, range of operating conditions, required pretreatment, and the most likely modes of failure. It is also necessary to demonstrate the effectiveness of the monitoring scheme and to establish the documentation and qualification requirements for the system's validation maintenance. Trials conducted in a pilot installation can be valuable in defining the operating parameters and the expected water quality and in identifying failure modes. However, qualification of the specific unit operation can only be performed as part of the validation of the installed operational system. The selection of specific unit operations and design characteristics for a water system should take into account the quality of the feed water, the technology chosen for subsequent processing steps, the extent and complexity of the water distribution system, and the appropriate compendial requirements. For example, in the design of a system for *Water for Injection*, the final process (distillation or whatever other validated process is used according to the monograph) must have effective bacterial endotoxin reduction capability and must be validated.

## UNIT OPERATIONS CONCERNS

The following is a brief description of selected unit operations and the operation and validation concerns associated with them. Not all unit operations are discussed, nor are all potential problems addressed. The purpose is to highlight issues that focus on the design, installation, operation, maintenance, and monitoring parameters that facilitate water system validation.

### Prefiltration

The purpose of prefiltration—also referred to as initial, coarse, or depth filtration—is to remove solid contaminants down to a size of 7 to 10  $\mu\text{m}$  from the incoming source water supply and protect downstream system components from particulates that can inhibit equipment performance and shorten their effective life. This coarse filtration technology

utilizes primarily sieving effects for particle capture and a depth of filtration medium that has a high “dirt load” capacity. Such filtration units are available in a wide range of designs and for various applications. Removal efficiencies and capacities differ significantly, from granular bed filters such as multimedia or sand for larger water systems, to depth cartridges for smaller water systems. Unit and system configurations vary widely in type of filtering media and location in the process. Granular or cartridge prefilters are often situated at or near the head of the water pretreatment system prior to unit operations designed to remove the source water disinfectants. This location, however, does not preclude the need for periodic microbial control because biofilm can still proliferate, although at a slower rate in the presence of source water disinfectants. Design and operational issues that may impact performance of depth filters include channeling of the filtering media, blockage from silt, microbial growth, and filtering-media loss during improper backwashing. Control measures involve pressure and flow monitoring during use and backwashing, sanitizing, and replacing filtering media. An important design concern is sizing of the filter to prevent channeling or media loss resulting from inappropriate water flow rates as well as proper sizing to minimize excessively frequent or infrequent backwashing or cartridge filter replacement.

### **Activated Carbon**

Granular activated carbon beds adsorb low molecular weight organic material and oxidizing additives, such as chlorine and chloramine compounds, removing them from the water. They are used to achieve certain quality attributes and to protect against reaction with downstream stainless steel surfaces, resins, and membranes. The chief operating concerns regarding activated carbon beds include the propensity to support bacteria growth, the potential for hydraulic channeling, the organic adsorption capacity, appropriate water flow rates and contact time, the inability to be regenerated in situ, and the shedding of bacteria, endotoxins, organic chemicals, and fine carbon particles. Control measures may involve monitoring water flow rates and differential pressures, sanitizing with hot water or steam, backwashing, testing for adsorption capacity, and frequent replacement of the carbon bed. If the activated carbon bed is intended for organic reduction, it may also be appropriate to monitor influent and effluent TOC. It is important to note that the use of steam for carbon bed sanitization is often incompletely effective due to steam channeling rather than even permeation through the bed. This phenomenon can usually be avoided by using hot water sanitization. It is also important to note that microbial biofilm development on the surface of the granular carbon particles (as well as on other particles such as found in deionizer beds and even multimedia beds) can cause adjacent bed granules to “stick” together. When large masses of granules are agglomerated in this fashion, normal backwashing and bed fluidization flow parameters may not be sufficient to disperse them, leading to ineffective removal of trapped debris, loose biofilm, and penetration of microbial controlling conditions (as well

as regenerant chemicals as in the case of agglomerated deionizer resins). Alternative technologies to activated carbon beds can be used in order to avoid their microbial problems, such as disinfectant-neutralizing chemical additives and regenerable organic scavenging devices. However, these alternatives do not function by the same mechanisms as activated carbon, may not be as effective at removing disinfectants and some organics, and have a different set of operating concerns and control measures that may be nearly as troublesome as activated carbon beds.

### **Additives**

Chemical additives are used in water systems (a) to control microorganisms by use of sanitants such as chlorine compounds and ozone, (b) to enhance the removal of suspended solids by use of flocculating agents, (c) to remove chlorine compounds, (d) to avoid scaling on reverse osmosis membranes, and (e) to adjust pH for more effective removal of carbonate and ammonia compounds by reverse osmosis. These additives do not constitute “added substances” as long as they are either removed by subsequent processing steps or are otherwise absent from the finished water. Control of additives to ensure a continuously effective concentration and subsequent monitoring to ensure their removal should be designed into the system and included in the monitoring program.

### **Organic Scavengers**

Organic scavenging devices use macroreticular weakly basic anion-exchange resins capable of removing organic material and endotoxins from the water. They can be regenerated with appropriate biocidal caustic brine solutions. Operating concerns are associated with organic scavenging capacity, particulate, chemical and microbiological fouling of the reactive resin surface, flow rate, regeneration frequency, and shedding of resin fragments. Control measures include TOC testing of influent and effluent, backwashing, monitoring hydraulic performance, and using downstream filters to remove resin fines.

### **Softeners**

Water softeners may be located either upstream or downstream of disinfectant removal units. They utilize sodium-based cation-exchange resins to remove water-hardness ions, such as calcium and magnesium, that could foul or interfere with the performance of downstream processing equipment such as reverse osmosis membranes, deionization devices, and distillation units. Water softeners can also be used to remove other lower affinity cations, such as the ammonium ion, that may be released from chloramine disinfectants commonly used in drinking water and which might otherwise carryover through other downstream unit operations. If ammonium removal is one of its purposes, the softener must be located downstream of the disinfectant removal operation, which itself may liberate ammonium from neutralized chloramine disinfectants. Water softener resin beds are regenerated with concentrated sodium chloride solution (brine). Concerns

include microorganism proliferation, channeling caused by biofilm agglomeration of resin particles, appropriate water flow rates and contact time, ion-exchange capacity, organic and particulate resin fouling, organic leaching from new resins, fracture of the resin beads, resin degradation by excessively chlorinated water, and contamination from the brine solution used for regeneration. Control measures involve recirculation of water during periods of low water use, periodic sanitization of the resin and brine system, use of microbial control devices (e.g., UV light and chlorine), locating the unit upstream of the disinfectant removal step (if used only for softening), appropriate regeneration frequency, effluent chemical monitoring (e.g., hardness ions and possibly ammonium), and downstream filtration to remove resin fines. If a softener is used for ammonium removal from chloramine-containing source water, then capacity, contact time, resin surface fouling, pH, and regeneration frequency are very important.

### Deionization

Deionization (DI), and continuous electrodeionization (CEDI) are effective methods of improving the chemical quality attributes of water by removing cations and anions. DI systems have charged resins that require periodic regeneration with an acid and base. Typically, cationic resins are regenerated with either hydrochloric or sulfuric acid, which replace the captured positive ions with hydrogen ions. Anionic resins are regenerated with sodium or potassium hydroxide, which replace captured negative ions with hydroxide ions. Because free endotoxin is negatively charged, there is some removal of endotoxin achieved by the anionic resin. Both regenerant chemicals are biocidal and offer a measure of microbial control. The system can be designed so that the cation and anion resins are in separate or “twin” beds or they can be mixed together to form a mixed bed. Twin beds are easily regenerated but deionize water less efficiently than mixed beds, which have a considerably more complex regeneration process. Rechargeable resin canisters can also be used for this purpose.

The CEDI system uses a combination of mixed resin, selectively permeable membranes, and an electric charge, providing continuous flow (product and waste concentrate) and continuous regeneration. Water enters both the resin section and the waste (concentrate) section. As it passes through the resin, it is deionized to become product water. The resin acts as a conductor enabling the electrical potential to drive the captured cations and anions through the resin and appropriate membranes for concentration and removal in the waste water stream. The electrical potential also separates the water in the resin (product) section into hydrogen and hydroxide ions. This permits continuous regeneration of the resin without the need for regenerant additives. However, unlike conventional deionization, CEDI units must start with water that is already partially purified because they generally cannot produce *Purified Water* quality when starting with the heavier ion load of unpurified source water.

Concerns for all forms of deionization units include microbial and endotoxin control,

chemical additive impact on resins and membranes, and loss, degradation, and fouling of resin. Issues of concern specific to DI units include regeneration frequency and completeness, channeling caused by biofilm agglomeration of resin particles, organic leaching from new resins, complete resin separation for mixed bed regeneration, and mixing air contamination (mixed beds). Control measures vary but typically include recirculation loops, effluent microbial control by UV light, conductivity monitoring, resin testing, microporous filtration of mixing air, microbial monitoring, frequent regeneration to minimize and control microorganism growth, sizing the equipment for suitable water flow and contact time, and use of elevated temperatures. Internal distributor and regeneration piping for mixed bed units should be configured to ensure that regeneration chemicals contact all internal bed and piping surfaces and resins. Rechargeable canisters can be the source of contamination and should be carefully monitored. Full knowledge of previous resin use, minimum storage time between regeneration and use, and appropriate sanitizing procedures are critical factors ensuring proper performance.

### **Reverse Osmosis**

Reverse osmosis (RO) units employ semipermeable membranes. The “pores” of RO membranes are actually intersegmental spaces among the polymer molecules. They are big enough for permeation of water molecules, but too small to permit passage of hydrated chemical ions. However, many factors including pH, temperature, and differential pressure across the membrane affect the selectivity of this permeation. With the proper controls, RO membranes can achieve chemical, microbial, and endotoxin quality improvement. The process streams consist of supply water, product water (permeate), and wastewater (reject). Depending on source water, pretreatment and system configuration variations and chemical additives may be necessary to achieve desired performance and reliability.

A major factor affecting RO performance is the permeate recovery rate, that is, the amount of the water passing through the membrane compared to the amount rejected. This is influenced by the several factors, but most significantly by the pump pressure. Recoveries of 75% are typical, and can accomplish a 1 to 2 log purification of most impurities. For most feed waters, this is usually not enough to meet *Purified Water* conductivity specifications. A second pass of this permeate water through another RO stage usually achieves the necessary permeate purity if other factors such as pH and temperature have been appropriately adjusted and the ammonia from chloraminated source water has been previously removed. Increasing recoveries with higher pressures in order to reduce the volume of reject water will lead to reduced permeate purity. If increased pressures are needed over time to achieve the same permeate flow, this is an indication of partial membrane blockage that needs to be corrected before it becomes irreversibly fouled, and expensive membrane replacement is the only option.

Other concerns associated with the design and operation of RO units include membrane



materials that are extremely sensitive to sanitizing agents and to particulate, chemical, and microbial membrane fouling; membrane and seal integrity; the passage of dissolved gases, such as carbon dioxide and ammonia; and the volume of wastewater, particularly where water discharge is tightly regulated by local authorities. Failure of membrane or seal integrity will result in product water contamination. Methods of control involve suitable pretreatment of the influent water stream, appropriate membrane material selection, integrity challenges, membrane design and heat tolerance, periodic sanitization, and monitoring of differential pressures, conductivity, microbial levels, and TOC.

The development of RO units that can tolerate sanitizing water temperatures as well as operate efficiently and continuously at elevated temperatures has added greatly to their microbial control and to the avoidance of biofouling. RO units can be used alone or in combination with DI and CEDI units as well as ultrafiltration for operational and quality enhancements.

### **Ultrafiltration**

Ultrafiltration is a technology most often employed in pharmaceutical water systems for removing endotoxins from a water stream. It can also use semipermeable membranes, but unlike RO, these typically use polysulfone membranes whose intersegmental “pores” have been purposefully exaggerated during their manufacture by preventing the polymer molecules from reaching their smaller equilibrium proximities to each other. Depending on the level of equilibrium control during their fabrication, membranes with differing molecular weight “cutoffs” can be created such that molecules with molecular weights above these cutoffs ratings are rejected and cannot penetrate the filtration matrix.

Ceramic ultrafilters are another molecular sieving technology. Ceramic ultrafilters are self supporting and extremely durable, backwashable, chemically cleanable, and steam sterilizable. However, they may require higher operating pressures than membrane type ultrafilters.

All ultrafiltration devices work primarily by a molecular sieving principle. Ultrafilters with molecular weight cutoff ratings in the range of 10,000 to 20,000 Da are typically used in water systems for removing endotoxins. This technology may be appropriate as an intermediate or final purification step. Similar to RO, successful performance is dependent upon pretreatment of the water by upstream unit operations.

Issues of concern for ultrafilters include compatibility of membrane material with heat and sanitizing agents, membrane integrity, fouling by particles and microorganisms, and seal integrity. Control measures involve filtration medium selection, sanitization, flow design (dead end vs. tangential), integrity challenges, regular cartridge changes, elevated feed water temperature, and monitoring TOC and differential pressure. Additional flexibility in operation is possible based on the way ultrafiltration units are arranged such as in a

parallel or series configurations. Care should be taken to avoid stagnant water conditions that could promote microorganism growth in back-up or standby units.

### **Charge-Modified Filtration**

Charge-modified filters are usually microbially retentive filters that are treated during their manufacture to have a positive charge on their surfaces. Microbial retentive filtration will be described in a subsequent section, but the significant feature of these membranes is their electrostatic surface charge. Such charged filters can reduce endotoxin levels in the fluids passing through them by their adsorption (owing to endotoxin's negative charge) onto the membrane surfaces. Though ultrafilters are more often employed as a unit operation for endotoxin removal in water systems, charge-modified filters may also have a place in endotoxin removal particularly where available upstream pressures are not sufficient for ultrafiltration and for a single, relatively short term use. Charge-modified filters may be difficult to validate for long-term or large-volume endotoxin retention. Even though their purified standard endotoxin retention can be well characterized, their retention capacity for “natural” endotoxins is difficult to gauge. Nevertheless, utility could be demonstrated and validated as short-term, single-use filters at points of use in water systems that are not designed for endotoxin control or where only an endotoxin “polishing” (removal of only slight or occasional endotoxin levels) is needed. Control and validation concerns include volume and duration of use, flow rate, water conductivity and purity, and constancy and concentration of endotoxin levels being removed. All of these factors may have to be evaluated and challenged prior to using this approach, making this a difficult-to-validate application. Even so, there may still be a possible need for additional backup endotoxin testing both upstream and downstream of the filter.

### **Microbial-Retentive Filtration**

Microbial-retentive membrane filters have experienced an evolution of understanding in the past decade that has caused previously held theoretical retention mechanisms to be reconsidered. These filters have a larger effective “pore size” than ultrafilters and are intended to prevent the passage of microorganisms and similarly sized particles without unduly restricting flow. This type of filtration is widely employed within water systems for filtering the bacteria out of both water and compressed gases as well as for vent filters on tanks and stills and other unit operations. However, the properties of the water system microorganisms seem to challenge a filter's microbial retention from water with phenomena absent from other aseptic filtration applications, such as filter sterilizing of pharmaceutical formulations prior to packaging. In the latter application, sterilizing grade filters are generally considered to have an assigned rating of 0.2 or 0.22  $\mu\text{m}$ . This rather arbitrary rating is associated with filters that have the ability to retain a high level challenge of a specially prepared inoculum of *Brevundimonas* (formerly *Pseudomonas*) *diminuta*. This is a small microorganism originally isolated decades ago from a product

that had been “filter sterilized” using a 0.45- $\mu\text{m}$  rated filter. Further study revealed that a percentage of cells of this microorganism could reproducibly penetrate the 0.45- $\mu\text{m}$  sterilizing filters. Through historic correlation of *B. diminuta* retaining tighter filters, thought to be twice as good as 0.45- $\mu\text{m}$  filter, assigned ratings of 0.2 or 0.22  $\mu\text{m}$  with their successful use in product solution filter sterilization, both this filter rating and the associated high level *B. diminuta* challenge have become the current benchmarks for sterilizing filtration. New evidence now suggests that for microbial-retentive filters used for pharmaceutical water, *B. diminuta* may not be the best model microorganism.

An archaic understanding of microbial retentive filtration would lead one to equate a filter's rating with the false impression of a simple sieve or screen that absolutely retains particles sized at or above the filter's rating. A current understanding of the mechanisms involved in microbial retention and the variables that can affect those mechanisms has yielded a far more complex interaction of phenomena than previously understood. A combination of simple sieve retention and surface adsorption are now known to contribute to microbial retention.

The following all interact to create some unusual and surprising retention phenomena for water system microorganisms: the variability in the range and average pore sizes created by the various membrane fabrication processes, the variability of the surface chemistry and three-dimensional structure related to the different polymers used in these filter matrices, and the size and surface properties of the microorganism intended to be retained by the filters. *B. diminuta* may not be the best challenge microorganisms for demonstrating bacterial retention for 0.2- to 0.22- $\mu\text{m}$  rated filters for use in water systems because it appears to be more easily retained by these filters than some water system flora. The well-documented appearance of water system microorganisms on the downstream sides of some 0.2- to 0.22- $\mu\text{m}$  rated filters after a relatively short period of use seems to support that some penetration phenomena are at work. Unknown for certain is if this downstream appearance is caused by a “blow-through” or some other pass-through phenomenon as a result of tiny cells or less cell “stickiness”, or by a “growth through” phenomenon as a result of cells hypothetically replicating their way through the pores to the downstream side. Whatever is the penetration mechanism, 0.2- to 0.22- $\mu\text{m}$  rated membranes may not be the best choice for some water system uses.

Microbial retention success in water systems has been reported with the use of some manufacturers' filters arbitrarily rated as 0.1  $\mu\text{m}$ . There is general agreement that for a given manufacturer, their 0.1- $\mu\text{m}$  rated filters are tighter than their 0.2- to 0.22- $\mu\text{m}$  rated filters. However, comparably rated filters from different manufacturers in water filtration applications may not perform equivalently owing to the different filter fabrication processes and the nonstandardized microbial retention challenge processes currently used for defining the 0.1- $\mu\text{m}$  filter rating. It should be noted that use of 0.1- $\mu\text{m}$  rated membranes generally results in a sacrifice in flow rate compared to 0.2- to 0.22- $\mu\text{m}$

membranes, so whatever membranes are chosen for a water system application, the user must verify that the membranes are suitable for their intended application, use period, and use process, including flow rate.

For microbial retentive gas filtrations, the same sieving and adsorptive retention phenomena are at work as in liquid filtration, but the adsorptive phenomenon is enhanced by additional electrostatic interactions between particles and filter matrix. These electrostatic interactions are so strong that particle retention for a given filter rating is significantly more efficient in gas filtration than in water or product solution filtrations. These additional adsorptive interactions render filters rated at 0.2 to 0.22  $\mu\text{m}$  unquestionably suitable for microbial retentive gas filtrations. When microbially retentive filters are used in these applications, the membrane surface is typically hydrophobic (non-wettable by water). A significant area of concern for gas filtration is blockage of tank vents by condensed water vapor, which can cause mechanical damage to the tank. Control measures include electrical or steam tracing and a self-draining orientation of vent filter housings to prevent accumulation of vapor condensate. However, a continuously high filter temperature will take an oxidative toll on polypropylene components of the filter, so sterilization of the unit prior to initial use, and periodically thereafter, as well as regular visual inspections, integrity tests, and changes are recommended control methods.

In water applications, microbial retentive filters may be used downstream of unit operations that tend to release microorganisms or upstream of unit operations that are sensitive to microorganisms. Microbial retentive filters may also be used to filter water feeding the distribution system. It should be noted that regulatory authorities allow the use of microbial retentive filters within distribution systems or even at use points if they have been properly validated and are appropriately maintained. A point-of-use filter should only be intended to “polish” the microbial quality of an otherwise well-maintained system and not to serve as the primary microbial control device. The efficacy of system microbial control measures can only be assessed by sampling the water upstream of the filters. As an added measure of protection, in-line UV lamps, appropriately sized for the flow rate (see *Sanitization*), may be used just upstream of microbial retentive filters to inactivate microorganisms prior to their capture by the filter. This tandem approach tends to greatly delay potential microbial penetration phenomena and can substantially extend filter service life.

### **Ultraviolet Light**

The use of low-pressure UV lights that emit a 254-nm wavelength for microbial control is discussed under *Sanitization*, but the application of UV light in chemical purification is also emerging. This 254-nm wavelength is also useful in the destruction of ozone. With intense emissions at wavelengths around 185 nm (as well as at 254 nm), medium pressure UV lights have demonstrated utility in the destruction of the chlorine-containing

disinfectants used in source water as well as for interim stages of water pretreatment.

High intensities of this wavelength alone or in combination with other oxidizing sanitants, such as hydrogen peroxide, have been used to lower TOC levels in recirculating distribution systems. The organics are typically converted to carbon dioxide, which equilibrates to bicarbonate, and incompletely oxidized carboxylic acids, both of which can easily be removed by polishing ion-exchange resins. Areas of concern include adequate UV intensity and residence time, gradual loss of UV emissivity with bulb age, gradual formation of UV-absorbing film at the water contact surface, incomplete photodegradation during unforeseen source water hyperchlorination, release of ammonia from chloramine photodegradation, unapparent UV bulb failure, and conductivity degradation in distribution systems using 185-nm UV lights. Control measures include regular inspection or emissivity alarms to detect bulb failures or film occlusions, regular UV bulb sleeve cleaning and wiping, downstream chlorine detectors, downstream polishing deionizers, and regular (approximately yearly) bulb replacement.

### **Distillation**

Distillation units provide chemical and microbial purification via thermal vaporization, mist elimination, and water vapor condensation. A variety of designs is available including single effect, multiple effect, and vapor compression. The latter two configurations are normally used in larger systems because of their generating capacity and efficiency. Distilled water systems require different feed water controls than required by membrane systems. For distillation, due consideration must be given to prior removal of hardness and silica impurities that may foul or corrode the heat transfer surfaces as well as prior removal of those impurities that could volatilize and condense along with the water vapor. In spite of general perceptions, even the best distillation process cannot afford absolute removal of contaminating ions and endotoxin. Most stills are recognized as being able to accomplish at least a 3 to 4 log reduction in these impurity concentrations. Areas of concern include carry-over of volatile organic impurities such as trihalomethanes (see *Source and Feed Water Considerations*) and gaseous impurities such as ammonia and carbon dioxide, faulty mist elimination, evaporator flooding, inadequate blowdown, stagnant water in condensers and evaporators, pump and compressor seal design, pinhole evaporator and condenser leaks, and conductivity (quality) variations during start-up and operation.

Methods of control may involve preliminary decarbonation steps to remove both dissolved carbon dioxide and other volatile or noncondensable impurities; reliable mist elimination to minimize feedwater droplet entrainment; visual or automated high water level indication to detect boiler flooding and boil over; use of sanitary pumps and compressors to minimize microbial and lubricant contamination of feedwater and condensate; proper drainage during inactive periods to minimize microbial growth and accumulation of associated endotoxin in boiler water; blow down control to limit the impurity

concentration effect in the boiler to manageable levels; on-line conductivity sensing with automated diversion to waste to prevent unacceptable water upon still startup or still malfunction from getting into the finished water distribute system; and periodic integrity testing for pinhole leaks to routinely assure condensate is not compromised by nonvolatized source water contaminants.

### **Storage Tanks**

Storage tanks are included in water distribution systems to optimize processing equipment capacity. Storage also allows for routine maintenance within the pretreatment train while maintaining continuous supply to meet manufacturing needs. Design and operation considerations are needed to prevent or minimize the development of biofilm, to minimize corrosion, to aid in the use of chemical sanitization of the tanks, and to safeguard mechanical integrity. These considerations may include using closed tanks with smooth interiors, the ability to spray the tank headspace using sprayballs on recirculating loop returns, and the use of heated, jacketed/insulated tanks. This minimizes corrosion and biofilm development and aids in thermal and chemical sanitization. Storage tanks require venting to compensate for the dynamics of changing water levels. This can be accomplished with a properly oriented and heat-traced filter housing fitted with a hydrophobic microbial retentive membrane filter affixed to an atmospheric vent. Alternatively, an automatic membrane-filtered compressed gas blanketing system may be used. In both cases, rupture disks equipped with a rupture alarm device should be used as a further safeguard for the mechanical integrity of the tank. Areas of concern include microbial growth or corrosion due to irregular or incomplete sanitization and microbial contamination from unalarmed rupture disk failures caused by condensate-occluded vent filters.

### **Distribution Systems**

Distribution system configuration should allow for the continuous flow of water in the piping by means of recirculation. Use of nonrecirculating, dead-end, or one-way systems or system segments should be avoided whenever possible. If not possible, these systems should be periodically flushed and more closely monitored. Experience has shown that continuously recirculated systems are easier to maintain. Pumps should be designed to deliver fully turbulent flow conditions to facilitate thorough heat distribution (for hot water sanitized systems) as well as thorough chemical sanitant distribution. Turbulent flow also appear to either retard the development of biofilms or reduce the tendency of those biofilms to shed bacteria into the water. If redundant pumps are used, they should be configured and used to avoid microbial contamination of the system. Components and distribution lines should be sloped and fitted with drain points so that the system can be completely drained. In stainless steel distribution systems where the water is circulated at a high temperature, dead legs and low-flow conditions should be

avoided, and valved tie-in points should have length-to-diameter ratios of six or less. If constructed of heat tolerant plastic, this ratio should be even less to avoid cool points where biofilm development could occur. In ambient temperature distribution systems, particular care should be exercised to avoid or minimize dead leg ratios of any size and provide for complete drainage. If the system is intended to be steam sanitized, careful sloping and low-point drainage is crucial to condensate removal and sanitization success. If drainage of components or distribution lines is intended as a microbial control strategy, they should also be configured to be completely dried using dry compressed air (or nitrogen if appropriate employee safety measures are used). Drained but still moist surfaces will still support microbial proliferation. Water exiting from the distribution system should not be returned to the system without first passing through all or a portion of the purification train.

The distribution design should include the placement of sampling valves in the storage tank and at other locations, such as in the return line of the recirculating water system. Where feasible, the primary sampling sites for water should be the valves that deliver water to the points of use. Direct connections to processes or auxiliary equipment should be designed to prevent reverse flow into the controlled water system. Hoses and heat exchangers that are attached to points of use in order to deliver water for a particular use must not chemically or microbiologically degrade the water quality. The distribution system should permit sanitization for microorganism control. The system may be continuously operated at sanitizing conditions or sanitized periodically.

## **INSTALLATION, MATERIALS OF CONSTRUCTION, AND COMPONENT SELECTION**

Installation techniques are important because they can affect the mechanical, corrosive, and sanitary integrity of the system. Valve installation attitude should promote gravity drainage. Pipe supports should provide appropriate slopes for drainage and should be designed to support the piping adequately under worst-case thermal and flow conditions. The methods of connecting system components including units of operation, tanks, and distribution piping require careful attention to preclude potential problems. Stainless steel welds should provide reliable joints that are internally smooth and corrosion-free. Low-carbon stainless steel, compatible wire filler, where necessary, inert gas, automatic welding machines, and regular inspection and documentation help to ensure acceptable weld quality. Follow-up cleaning and passivation are important for removing contamination and corrosion products and to re-establish the passive corrosion resistant surface. Plastic materials can be fused (welded) in some cases and also require smooth, uniform internal surfaces. Adhesive glues and solvents should be avoided due to the potential for voids and extractables. Mechanical methods of joining, such as flange fittings, require care to avoid the creation of offsets, gaps, penetrations, and voids. Control measures include good alignment, properly sized gaskets, appropriate spacing,

uniform sealing force, and the avoidance of threaded fittings.

Materials of construction should be selected to be compatible with control measures such as sanitizing, cleaning, and passivating. Temperature rating is a critical factor in choosing appropriate materials because surfaces may be required to handle elevated operating and sanitization temperatures. Should chemicals or additives be used to clean, control, or sanitize the system, materials resistant to these chemicals or additives must be utilized. Materials should be capable of handling turbulent flow and elevated velocities without wear of the corrosion-resistant film such as the passive chromium oxide surface of stainless steel. The finish on metallic materials such as stainless steel, whether it is a refined mill finish, polished to a specific grit, or an electropolished treatment, should complement system design and provide satisfactory corrosion and microbial activity resistance as well as chemical sanitizability. Auxiliary equipment and fittings that require seals, gaskets, diaphragms, filter media, and membranes should exclude materials that permit the possibility of extractables, shedding, and microbial activity. Insulating materials exposed to stainless steel surfaces should be free of chlorides to avoid the phenomenon of stress corrosion cracking that can lead to system contamination and the destruction of tanks and critical system components.

Specifications are important to ensure proper selection of materials and to serve as a reference for system qualification and maintenance. Information such as mill reports for stainless steel and reports of composition, ratings, and material handling capabilities for nonmetallic substances should be reviewed for suitability and retained for reference. Component (auxiliary equipment) selection should be made with assurance that it does not create a source of contamination intrusion. Heat exchangers should be constructed to prevent leakage of heat transfer medium to the pharmaceutical water and, for heat exchanger designs where prevention may fail, there should be a means to detect leakage. Pumps should be of sanitary design with seals that prevent contamination of the water. Valves should have smooth internal surfaces with the seat and closing device exposed to the flushing action of water, such as occurs in diaphragm valves. Valves with pocket areas or closing devices (e.g., ball, plug, gate, globe) that move into and out of the flow area should be avoided.

## **SANITIZATION**

Microbial control in water systems is achieved primarily through sanitization practices.

Systems can be sanitized using either thermal or chemical means. Thermal approaches to system sanitization include periodic or continuously circulating hot water and the use of steam. Temperatures of at least 80° are most commonly used for this purpose, but continuously recirculating water of at least 65° has also been used effectively in insulated stainless steel distribution systems when attention is paid to uniformity and distribution of such self-sanitizing temperatures. These techniques are limited to systems



that are compatible with the higher temperatures needed to achieve sanitization. Although thermal methods control biofilm development by either continuously inhibiting their growth or, in intermittent applications, by killing the microorganisms within biofilms, they are not effective in removing established biofilms. Killed but intact biofilms can become a nutrient source for rapid biofilm regrowth after the sanitizing conditions are removed or halted. In such cases, a combination of routine thermal and periodic supplementation with chemical sanitization might be more effective. The more frequent the thermal sanitization, the more likely biofilm development and regrowth can be eliminated. Chemical methods, where compatible, can be used on a wider variety of construction materials. These methods typically employ oxidizing agents such as halogenated compounds, hydrogen peroxide, ozone, peracetic acid, or combinations thereof. Halogenated compounds are effective sanitizers but are difficult to flush from the system and may leave biofilms intact. Compounds such as hydrogen peroxide, ozone, and peracetic acid oxidize bacteria and biofilms by forming reactive peroxides and free radicals (notably hydroxyl radicals). The short half-life of ozone in particular, and its limitation on achievable concentrations require that it be added continuously during the sanitization process. Hydrogen peroxide and ozone rapidly degrade to water and oxygen; peracetic acid degrades to acetic acid in the presence of UV light. In fact, ozone's ease of degradation to oxygen using 254-nm UV lights at use points allow it to be most effectively used on a continuous basis to provide continuously sanitizing conditions.

In-line UV light at a wavelength of 254 nm can also be used to continuously “sanitize” water circulating in the system, but these devices must be properly sized for the water flow. Such devices inactivate a high percentage (but not 100%) of microorganisms that flow through the device but cannot be used to directly control existing biofilm upstream or downstream of the device. However, when coupled with conventional thermal or chemical sanitization technologies or located immediately upstream of a microbially retentive filter, it is most effective and can prolong the interval between system sanitizations.

It is important to note that microorganisms in a well-developed biofilm can be extremely difficult to kill, even by aggressive oxidizing biocides. The less developed and therefore thinner the biofilm, the more effective the biocidal action. Therefore, optimal biocide control is achieved by frequent biocide use that does not allow significant biofilm development between treatments.

Sanitization steps require validation to demonstrate the capability of reducing and holding microbial contamination at acceptable levels. Validation of thermal methods should include a heat distribution study to demonstrate that sanitization temperatures are achieved throughout the system, including the body of use point valves. Validation of chemical methods require demonstrating adequate chemical concentrations throughout

the system, exposure to all wetted surfaces, including the body of use point valves, and complete removal of the sanitant from the system at the completion of treatment. Methods validation for the detection and quantification of residues of the sanitant or its objectionable degradants is an essential part of the validation program. The frequency of sanitization should be supported by, if not triggered by, the results of system microbial monitoring. Conclusions derived from trend analysis of the microbiological data should be used as the alert mechanism for maintenance. The frequency of sanitization should be established in such a way that the system operates in a state of microbiological control and does not routinely exceed alert levels (see *Alert and Action Levels and Specifications*).

## OPERATION, MAINTENANCE, AND CONTROL

A preventive maintenance program should be established to ensure that the water system remains in a state of control. The program should include (1) procedures for operating the system, (2) monitoring programs for critical quality attributes and operating conditions including calibration of critical instruments, (3) schedule for periodic sanitization, (4) preventive maintenance of components, and (5) control of changes to the mechanical system and to operating conditions.

**Operating Procedures**— Procedures for operating the water system and performing routine maintenance and corrective action should be written, and they should also define the point when action is required. The procedures should be well documented, detail the function of each job, assign who is responsible for performing the work, and describe how the job is to be conducted. The effectiveness of these procedures should be assessed during water system validation.

**Monitoring Program**— Critical quality attributes and operating parameters should be documented and monitored. The program may include a combination of in-line sensors or automated instruments (e.g., for TOC, conductivity, hardness, and chlorine), automated or manual documentation of operational parameters (such as flow rates or pressure drop across a carbon bed, filter, or RO unit), and laboratory tests (e.g., total microbial counts). The frequency of sampling, the requirement for evaluating test results, and the necessity for initiating corrective action should be included.

**Sanitization**— Depending on system design and the selected units of operation, routine periodic sanitization may be necessary to maintain the system in a state of microbial control. Technologies for sanitization are described above.

**Preventive Maintenance**— A preventive maintenance program should be in effect. The program should establish what preventive maintenance is to be performed, the frequency of maintenance work, and how the work should be documented.

**Change Control**— The mechanical configuration and operating conditions must be controlled. Proposed changes should be evaluated for their impact on the whole system. The need to requalify the system after changes are made should be determined. Following a decision to modify a water system, the affected drawings, manuals, and procedures should be revised.

## SAMPLING CONSIDERATIONS

Water systems should be monitored at a frequency that is sufficient to ensure that the system is in control and continues to produce water of acceptable quality. Samples should be taken from representative locations within the processing and distribution system. Established sampling frequencies should be based on system validation data and should cover critical areas including unit operation sites. The sampling plan should take into consideration the desired attributes of the water being sampled. For example, systems for *Water for Injection* because of their more critical microbiological requirements, may require a more rigorous sampling frequency.

Analyses of water samples often serve two purposes: in-process control assessments and final quality control assessments. In-process control analyses are usually focused on the attributes of the water within the system. Quality control is primarily concerned with the attributes of the water delivered by the system to its various uses. The latter usually employs some sort of transfer device, often a flexible hose, to bridge the gap between the distribution system use-point valve and the actual location of water use. The issue of sample collection location and sampling procedure is often hotly debated because of the typically mixed use of the data generated from the samples, for both in-process control and quality control. In these single sample and mixed data use situations, the worst-case scenario should be utilized. In other words, samples should be collected from use points using the same delivery devices, such as hoses, and procedures, such as preliminary hose or outlet flushing, as are employed by production from those use points. Where use points per se cannot be sampled, such as hard-piped connections to equipment, special sampling ports may be used. In all cases, the sample must represent as closely as possible the quality of the water used in production. If a point of use filter is employed, sampling of the water prior to and after the filter is needed because the filter will mask the microbial control achieved by the normal operating procedures of the system.

Samples containing chemical sanitizing agents require neutralization prior to microbiological analysis. Samples for microbiological analysis should be tested immediately, or suitably refrigerated to preserve the original microbial attributes until analysis can begin. Samples of flowing water are only indicative of the concentration of planktonic (free floating) microorganisms present in the system. Biofilm microorganisms (those attached to water system surfaces) are usually present in greater numbers and

are the source of the planktonic population recovered from grab samples. Microorganisms in biofilms represent a continuous source of contamination and are difficult to directly sample and quantify. Consequently, the planktonic population is usually used as an indicator of system contamination levels and is the basis for system *Alert* and *Action Levels*. The consistent appearance of elevated planktonic levels is usually an indication of advanced biofilm development in need of remedial control. System control and sanitization are key in controlling biofilm formation and the consequent planktonic population.

Sampling for chemical analyses is also done for in-process control and for quality control purposes. However, unlike microbial analyses, chemical analyses can be and often are performed using on-line instrumentation. Such on-line testing has unequivocal in-process control purposes because it is not performed on the water delivered from the system. However, unlike microbial attributes, chemical attributes are usually not significantly degraded by hoses. Therefore, through verification testing, it may be possible to show that the chemical attributes detected by the on-line instrumentation (in-process testing) are equivalent to those detected at the ends of the use point hoses (quality control testing). This again creates a single sample and mixed data use scenario. It is far better to operate the instrumentation in a continuous mode, generating large volumes of in-process data, but only using a defined small sampling of that data for QC purposes. Examples of acceptable approaches include using highest values for a given period, highest time-weighted average for a given period (from fixed or rolling sub-periods), or values at a fixed daily time. Each approach has advantages and disadvantages relative to calculation complexity and reflection of continuous quality, so the user must decide which approach is most suitable or justifiable.

## CHEMICAL CONSIDERATIONS

The chemical attributes of *Purified Water* and *Water for Injection* in effect prior to *USP 23* were specified by a series of chemistry tests for various specific and nonspecific attributes with the intent of detecting chemical species indicative of incomplete or inadequate purification. While these methods could have been considered barely adequate to control the quality of these waters, they nevertheless stood the test of time. This was partly because the operation of water systems was, and still is, based on on-line conductivity measurements and specifications generally thought to preclude the failure of these archaic chemistry attribute tests.

USP moved away from these chemical attribute tests to contemporary analytical technologies for the bulk waters *Purified Water* and *Water for Injection*. The intent was to upgrade the analytical technologies without tightening the quality requirements. The two contemporary analytical technologies employed were TOC and conductivity. The TOC test replaced the test for *Oxidizable substances* that primarily targeted organic

contaminants. A multistaged *Conductivity* test which detects ionic (mostly inorganic) contaminants replaced, with the exception of the test for *Heavy metals*, all of the inorganic chemical tests (i.e., *Ammonia*, *Calcium*, *Carbon dioxide*, *Chloride*, *Sulfate*).

Replacing the heavy metals attribute was considered unnecessary because (a) the source water specifications (found in the NPDWR) for individual *Heavy metals* were tighter than the approximate limit of detection of the *Heavy metals* test for *USP XXII Water for Injection* and *Purified Water* (approximately 0.1 ppm), (b) contemporary water system construction materials do not leach heavy metal contaminants, and (c) test results for this attribute have uniformly been negative—there has not been a confirmed occurrence of a singular test failure (failure of only the *Heavy metals* test with all other attributes passing) since the current heavy metal drinking water standards have been in place. Nevertheless, since the presence of heavy metals in *Purified Water* or *Water for Injection* could have dire consequences, its absence should at least be documented during new water system commissioning and validation or through prior test results records.

*Total solids* and *pH* were the only tests not covered by conductivity testing. The test for *Total solids* was considered redundant because the nonselective tests of conductivity and TOC could detect most chemical species other than silica, which could remain undetected in its colloidal form. Colloidal silica in *Purified Water* and *Water for Injection* is easily removed by most water pretreatment steps and even if present in the water, constitutes no medical or functional hazard except under extreme and rare situations. In such extreme situations, other attribute extremes are also likely to be detected. It is, however, the user's responsibility to ensure fitness for use. If silica is a significant component in the source water, and the purification unit operations could be operated or fail and selectively allow silica to be released into the finished water (in the absence of co-contaminants detectable by conductivity), then either silica-specific or a total solids type testing should be utilized to monitor and control this rare problem.

The *pH* attribute was eventually recognized to be redundant to the conductivity test (which included *pH* as an aspect of the test and specification); therefore, *pH* was dropped as a separate attribute test.

The rationale used by USP to establish its *Purified Water* and *Water for Injection* conductivity specifications took into consideration the conductivity contributed by the two least conductive former attributes of *Chloride* and *Ammonia*, thereby precluding their failure had those wet chemistry tests been performed. In essence, the *Stage 3* conductivity specifications (see [Water Conductivity, Bulk Water](#) { 645 }) were established from the sum of the conductivities of the limit concentrations of chloride ions (from pH 5.0 to 6.2) and ammonia ions (from pH 6.3 to 7.0), plus the unavoidable contribution of other conductivity-contributing ions from water ( $H^+$  and  $OH^-$ ), dissolved atmospheric  $CO_2$  (as  $HCO_3^-$ ), and an electro-balancing quantity of either  $Na^+$  or  $Cl^-$ ,

depending on the pH-induced ionic imbalance (see [Table 1](#)). The *Stage 2* conductivity specification is the lowest value on this table, 2.1  $\mu\text{S}/\text{cm}$ . The *Stage 1* specifications, designed primarily for on-line measurements, were derived essentially by summing the lowest values in the contributing ion columns for each of a series of tables similar to [Table 1](#), created for each 5° increment between 0° and 100°. For example purposes, the italicized values in [Table 1](#), the conductivity data table for 25°, were summed to yield a conservative value of 1.3  $\mu\text{S}/\text{cm}$ , the *Stage 1* specification for a nontemperature compensated, nonatmosphere equilibrated water sample that actually had a measured temperature of 25° to 29°. Each 5° increment in the table was similarly treated to yield the individual values listed in the table of *Stage 1* specifications (see [Water Conductivity, Bulk Water](#) { 645 } ).

**Table 1. Contributing Ion Conductivities of the Chloride - Ammonia Model as a Function of pH**

(in atmosphere-equilibrated water at 25°)

				Conductivity ( $\mu\text{S}/\text{cm}$ )				
pH	H <sup>+</sup>	OH <sup>-</sup>	HCO <sub>3</sub> <sup>-</sup>	Cl <sup>-</sup>	Na <sup>+</sup>	NH <sub>4</sub> <sup>+</sup>	Combined Conductivities	Stage 3 Limit
5.0	3.49	0	0.02	1.01	0.19	0	4.71	4.7
5.1	2.77	0	0.02	1.01	0.29	0	4.09	4.1
5.2	2.20	0	0.03	1.01	0.38	0	3.62	3.6
5.3	1.75	0	0.04	1.01	0.46	0	3.26	3.3
5.4	1.39	0	0.05	1.01	0.52	0	2.97	3.0
5.5	1.10	0	0.06	1.01	0.58	0	2.75	2.8
5.6	0.88	0	0.08	1.01	0.63	0	2.60	2.6
5.7	0.70	0	0.10	1.01	0.68	0	2.49	2.5
5.8	0.55	0	0.12	1.01	0.73	0	2.41	2.4
5.9	0.44	0	0.16	1.01	0.78	0	2.39	2.4
6.0	0.35	0	0.20	1.01	0.84	0	2.40	2.4
6.1	0.28	0	0.25	1.01	0.90	0	2.44	2.4
6.2	0.22	0	0.31	1.01	0.99	0	2.53	2.5
6.3	0.18	0	0.39	0.63	0	1.22	2.42	2.4
6.4	0.14	0.01	0.49	0.45	0	1.22	2.31	2.3
6.5	0.11	0.01	0.62	0.22	0	1.22	2.18	2.2
6.6	0.09	0.01	0.78	0	0.04	1.22	2.14	2.1
6.7	0.07	0.01	0.99	0	0.27	1.22	2.56	2.6
6.8	0.06	0.01	1.24	0	0.56	1.22	3.09	3.1
6.9	0.04	0.02	1.56	0	0.93	1.22	3.77	3.8
7.0	0.03	0.02	1.97	0	1.39	1.22	4.63	4.6

As stated above, this rather radical change to utilizing a conductivity attribute as well as

the inclusion of a TOC attribute allowed for on-line measurements. This was a major philosophical change and allowed major savings to be realized by industry. The TOC and conductivity tests can also be performed “off-line” in the laboratories using collected samples, though sample collection tends to introduce opportunities for adventitious contamination that can cause false high readings. The collection of on-line data is not, however, without challenges. The continuous readings tend to create voluminous amounts of data where before only a single data point was available. As stated under *Sampling Considerations*, continuous in-process data is excellent for understanding how a water system performs during all of its various usage and maintenance events in real time, but is too much data for QC purposes. Therefore, a justifiable fraction or averaging of the data can be used that is still representative of the overall water quality being used.

Packaged/sterile waters present a particular dilemma relative to the attributes of conductivity and TOC. The package itself is the source of chemicals (inorganics and organics) that leach over time into the water and can easily be detected. The irony of organic leaching from plastic packaging is that when the *Oxidizable substances* test was the only “organic contaminant” test for both bulk and packaged/sterile waters, that test's insensitivity to those organic leachables allowed their presence in packaged/sterile water to be quite high (possibly many times the TOC specification for bulk water). Similarly, glass containers can also leach inorganics, such as sodium, which are easily detected by conductivity, but poorly detected by the former wet chemistry attribute tests. Most of these leachables are considered harmless by current perceptions and standards at the rather significant concentrations present. Nevertheless, they effectively degrade the quality of the high-purity waters placed into these packaging systems. Some packaging materials contain more leachables than others and may not be as suitable for holding water and maintaining its purity.

The attributes of conductivity and TOC tend to reveal more about the packaging leachables than they do about the water's original purity. These currently “allowed” leachables could render the packaged/sterile versions of originally equivalent bulk water essentially unsuitable for many uses where the bulk waters are perfectly adequate.

Therefore, to better control the ionic packaging leachables, [Water Conductivity](#) [645](#) is divided into two sections. The first is titled *Bulk Water*, which applies to *Purified Water*, *Water for Injection*, *Water for Hemodialysis*, and *Pure Steam*, and includes the three-stage conductivity testing instructions and specifications. The second is titled *Sterile Water*, which applies to *Sterile Purified Water*, *Sterile Water for Injection*, *Sterile Water for Inhalation*, and *Sterile Water for Irrigation*. The *Sterile Water* section includes conductivity specifications similar to the *Stage 2* testing approach because it is intended as a laboratory test, and these sterile waters were made from bulk water that already complied with the three-stage conductivity test. In essence, packaging leachables are

the primary target “analytes” of the conductivity specifications in the *Sterile Water* section of [Water Conductivity](#) [〈 645 〉](#). The effect on potential leachables from different container sizes is the rationale for having two different specifications, one for small packages containing nominal volumes of 10 mL or less and another for larger packages. These conductivity specifications are harmonized with the *European Pharmacopoeia* conductivity specifications for *Sterile Water for Injection*. All monographed waters, except *Bacteriostatic Water for Injection*, have a conductivity specification that directs the user to either the *Bulk Water* or the *Sterile Water* section of [Water Conductivity](#) [〈 645 〉](#). For the sterile water monographs, this water conductivity specification replaces the redundant wet chemistry limit tests intended for inorganic contaminants that had previously been specified in these monographs.

### MICROBIAL CONSIDERATIONS

The major exogenous source of microbial contamination of bulk pharmaceutical water is source or feed water. Feed water quality must, at a minimum, meet the quality attributes of Drinking Water for which the level of coliforms are regulated. A wide variety of other microorganisms, chiefly Gram-negative bacteria, may be present in the incoming water. These microorganisms may compromise subsequent purification steps. Examples of other potential exogenous sources of microbial contamination include unprotected vents, faulty air filters, ruptured rupture disks, backflow from contaminated outlets, unsanitized distribution system “openings” including routine component replacements, inspections, repairs, and expansions, inadequate drain and air-breaks, and replacement activated carbon, deionizer resins, and regenerant chemicals. In these situations, the exogenous contaminants may not be normal aquatic bacteria but rather microorganisms of soil or even human origin. The detection of nonaquatic microorganisms may be an indication of a system component failure, which should trigger investigations that will remediate their source. Sufficient care should be given to system design and maintenance in order to minimize microbial contamination from these exogenous sources.

Unit operations can be a major source of endogenous microbial contamination.

Microorganisms present in feed water may adsorb to carbon bed, deionizer resins, filter membranes, and other unit operation surfaces and initiate the formation of a biofilm. In a high-purity water system, biofilm is an adaptive response by certain microorganisms to survive in this low nutrient environment. Downstream colonization can occur when microorganisms are shed from existing biofilm-colonized surfaces and carried to other areas of the water system. Microorganisms may also attach to suspended particles such as carbon bed fines or fractured resin particles. When the microorganisms become planktonic, they serve as a source of contamination to subsequent purification equipment (compromising its functionality) and to distribution systems.



Another source of endogenous microbial contamination is the distribution system itself.

Microorganisms can colonize pipe surfaces, rough welds, badly aligned flanges, valves, and unidentified dead legs, where they proliferate, forming a biofilm. The smoothness and composition of the surface may affect the rate of initial microbial adsorption, but once adsorbed, biofilm development, unless otherwise inhibited by sanitizing conditions, will occur regardless of the surface. Once formed, the biofilm becomes a continuous source of microbial contamination.

### ENDOTOXIN CONSIDERATIONS

Endotoxins are lipopolysaccharides found in and shed from the cell envelope that is external to the cell wall of Gram-negative bacteria. Gram-negative bacteria that form biofilms can become a source of endotoxins in pharmaceutical waters. Endotoxins may occur as clusters of lipopolysaccharide molecules associated with living microorganisms, fragments of dead microorganisms or the polysaccharide slime surrounding biofilm bacteria, or as free molecules. The free form of endotoxins may be released from cell surfaces of the bacteria that colonize the water system, or from the feed water that may enter the water system. Because of the multiplicity of endotoxin sources in a water system, endotoxin quantitation in a water system is not a good indicator of the level of biofilm abundance within a water system.

Endotoxin levels may be minimized by controlling the introduction of free endotoxins and microorganisms in the feed water and minimizing microbial proliferation in the system. This may be accomplished through the normal exclusion or removal action afforded by various unit operations within the treatment system as well as through system sanitization. Other control methods include the use of ultrafilters or charge-modified filters, either in-line or at the point of use. The presence of endotoxins may be monitored as described in the general test chapter [Bacterial Endotoxins Test](#) { 85 }.

### MICROBIAL ENUMERATION CONSIDERATIONS

The objective of a water system microbiological monitoring program is to provide sufficient information to control and assess the microbiological quality of the water produced.

Product quality requirements should dictate water quality specifications. An appropriate level of control may be maintained by using data trending techniques and, if necessary, limiting specific contraindicated microorganisms. Consequently, it may not be necessary to detect all of the microorganisms species present in a given sample. The monitoring program and methodology should indicate adverse trends and detect microorganisms that are potentially harmful to the finished product, process, or consumer. Final selection of method variables should be based on the individual requirements of the system being monitored.

It should be recognized that there is no single method that is capable of detecting all of the potential microbial contaminants of a water system. The methods used for microbial monitoring should be capable of isolating the numbers and types of organisms that have been deemed significant relative to in-process system control and product impact for each individual system. Several criteria should be considered when selecting a method to monitor the microbial content of a pharmaceutical water system. These include method sensitivity, range of organisms types or species recovered, sample processing throughput, incubation period, cost, and methodological complexity. An alternative consideration to the use of the classical “culture” approaches is a sophisticated instrumental or rapid test method that may yield more timely results. However, care must be exercised in selecting such an alternative approach to ensure that it has both sensitivity and correlation to classical culture approaches, which are generally considered the accepted standards for microbial enumeration.

Consideration should also be given to the timeliness of microbial enumeration testing after sample collection. The number of detectable planktonic bacteria in a sample collected in a scrupulously clean sample container will usually drop as time passes. The planktonic bacteria within the sample will tend to either die or to irretrievably adsorb to the container walls reducing the number of viable planktonic bacteria that can be withdrawn from the sample for testing. The opposite effect can also occur if the sample container is not scrupulously clean and contains a low concentration of some microbial nutrient that could promote microbial growth within the sample container. Because the number of recoverable bacteria in a sample can change positively or negatively over time after sample collection, it is best to test the samples as soon as possible after being collected. If it is not possible to test the sample within about 2 hours of collection, the sample should be held at refrigerated temperatures (2° to 8°) for a maximum of about 12 hours to maintain the microbial attributes until analysis. In situations where even this is not possible (such as when using off-site contract laboratories), testing of these refrigerated samples should be performed within 48 hours after sample collection. In the delayed testing scenario, the recovered microbial levels may not be the same as would have been recovered had the testing been performed shortly after sample collection. Therefore, studies should be performed to determine the existence and acceptability of potential microbial enumeration aberrations caused by protracted testing delays.

### **The Classical Culture Approach**

Classical culture approaches for microbial testing of water include but are not limited to pour plates, spread plates, membrane filtration, and most probable number (MPN) tests. These methods are generally easy to perform, are less expensive, and provide excellent sample processing throughput. Method sensitivity can be increased via the use of larger sample sizes. This strategy is used in the membrane filtration method. Culture approaches are further defined by the type of medium used in combination with the

incubation temperature and duration. This combination should be selected according to the monitoring needs presented by a specific water system as well as its ability to recover the microorganisms of interest: those that could have a detrimental effect on the product or process uses as well as those that reflect the microbial control status of the system.

There are two basic forms of media available for traditional microbiological analysis:

“high nutrient” and “low nutrient”. High-nutrient media such as plate count agar (TGYA) and m-HPC agar (formerly m-SPC agar), are intended as general media for the isolation and enumeration of heterotrophic or “copiotrophic” bacteria. Low-nutrient media such as R2A agar and NWRI agar (HPCA), may be beneficial for isolating slow growing “oligotrophic” bacteria and bacteria that require lower levels of nutrients to grow optimally. Often some facultative oligotrophic bacteria are able to grow on high nutrient media and some facultative copiotrophic bacteria are able to grow on low-nutrient media, but this overlap is not complete. Low-nutrient and high-nutrient cultural approaches may be concurrently used, especially during the validation of a water system, as well as periodically thereafter. This concurrent testing could determine if any additional numbers or types of bacteria can be preferentially recovered by one of the approaches. If so, the impact of these additional isolates on system control and the end uses of the water could be assessed. Also, the efficacy of system controls and sanitization on these additional isolates could be assessed.

Duration and temperature of incubation are also critical aspects of a microbiological test method. Classical methodologies using high nutrient media are typically incubated at 30° to 35° for 48 to 72 hours. Because of the flora in certain water systems, incubation at lower temperatures (e.g., 20° to 25°) for longer periods (e.g., 5 to 7 days) can recover higher microbial counts when compared to classical methods. Low-nutrient media are designed for these lower temperature and longer incubation conditions (sometimes as long as 14 days to maximize recovery of very slow growing oligotrophs or sanitant injured microorganisms), but even high-nutrient media can sometimes increase their recovery with these longer and cooler incubation conditions. Whether or not a particular system needs to be monitored using high- or low-nutrient media with higher or lower incubation temperatures or shorter or longer incubation times should be determined during or prior to system validation and periodically reassessed as the microbial flora of a new water system gradually establish a steady state relative to its routine maintenance and sanitization procedures. The establishment of a “steady state” can take months or even years and can be perturbed by a change in use patterns, a change in routine and preventative maintenance or sanitization procedures, and frequencies, or any type of system intrusion, such as for component replacement, removal, or addition. The decision to use longer incubation periods should be made after balancing the need for timely information and the type of corrective actions required when an alert or action level is exceeded with the ability to recover the microorganisms of interest.

The advantages gained by incubating for longer times, namely recovery of injured microorganisms, slow growers, or more fastidious microorganisms, should be balanced against the need to have a timely investigation and to take corrective action, as well as the ability of these microorganisms to detrimentally affect products or processes. In no case, however, should incubation at 30° to 35° be less than 48 hours or less than 96 hours at 20° to 25°.

Normally, the microorganisms that can thrive in extreme environments are best cultivated in the laboratory using conditions simulating the extreme environments from which they were taken. Therefore, thermophilic bacteria might be able to exist in the extreme environment of hot pharmaceutical water systems, and if so, could only be recovered and cultivated in the laboratory if similar thermal conditions were provided. Thermophilic aquatic microorganisms do exist in nature, but they typically derive their energy for growth from harnessing the energy from sunlight, from oxidation/reduction reactions of elements such as sulfur or iron, or indirectly from other microorganisms that do derive their energy from these processes. Such chemical/nutritional conditions do not exist in high purity water systems, whether ambient or hot. Therefore, it is generally considered pointless to search for thermophiles from hot pharmaceutical water systems owing to their inability to grow there.

The microorganisms that inhabit hot systems tend to be found in much cooler locations within these systems, for example, within use-point heat exchangers or transfer hoses. If this occurs, the kinds of microorganisms recovered are usually of the same types that might be expected from ambient water systems. Therefore, the mesophilic microbial cultivation conditions described later in this chapter are usually adequate for their recovery.

### **“Instrumental” Approaches**

Examples of instrumental approaches include microscopic visual counting techniques (e.g., epifluorescence and immunofluorescence) and similar automated laser scanning approaches and radiometric, impedometric, and biochemically based methodologies. These methods all possess a variety of advantages and disadvantages. Advantages could be their precision and accuracy or their speed of test result availability as compared to the classical cultural approach. In general, instrument approaches often have a shorter lead time for obtaining results, which could facilitate timely system control. This advantage, however, is often counterbalanced by limited sample processing throughput due to extended sample collection time, costly and/or labor-intensive sample processing, or other instrument and sensitivity limitations.

Furthermore, instrumental approaches are typically destructive, precluding subsequent isolate manipulation for characterization purposes. Generally, some form of microbial isolate characterization, if not full identification, may be a required element of water system monitoring. Consequently, culturing approaches have traditionally been preferred

over instrumental approaches because they offer a balance of desirable test attributes and post-test capabilities.

### Suggested Methodologies

The following general methods were originally derived from *Standard Methods for the Examination of Water and Wastewater*, 17<sup>th</sup> Edition, American Public Health Association, Washington, DC 20005. Even though this publication has undergone several revisions since its first citation in this chapter, the methods are still considered appropriate for establishing trends in the number of colony-forming units observed in the routine microbiological monitoring of pharmaceutical waters. It is recognized, however, that other combinations of media and incubation time and temperature may occasionally or even consistently result in higher numbers of colony-forming units being observed and/or different species being recovered.

The extended incubation periods that are usually required by some of the alternative methods available offer disadvantages that may outweigh the advantages of the higher counts that may be obtained. The somewhat higher baseline counts that might be observed using alternate cultural conditions would not necessarily have greater utility in detecting an excursion or a trend. In addition, some alternate cultural conditions using low-nutrient media tend to lead to the development of microbial colonies that are much less differentiated in colonial appearance, an attribute that microbiologists rely on when selecting representative microbial types for further characterization. It is also ironic that the nature of some of the slow growers and the extended incubation times needed for their development into visible colonies may also lead to those colonies being largely nonviable, which limits their further characterization and precludes their subculture and identification.

Methodologies that can be suggested as generally satisfactory for monitoring pharmaceutical water systems are as follows. However, it must be noted that these are not referee methods nor are they necessarily optimal for recovering microorganisms from all water systems. The users should determine through experimentation with various approaches which methodologies are best for monitoring their water systems for in-process control and quality control purposes as well as for recovering any contraindicated species they may have specified.

<i>Drinking Water:</i>	Pour Plate Method or Membrane Filtration Method <sup>1</sup>
	Sample Volume—1.0 mL minimum <sup>2</sup> Growth Medium—Plate Count Agar <sup>3</sup> Incubation Time—48 to 72 hours minimum Incubation Temperature—30° to 35°
<i>Purified Water:</i>	Pour Plate Method or Membrane Filtration Method <sup>1</sup>

	Sample Volume—1.0 mL minimum <sup>2</sup> Growth Medium—Plate Count Agar <sup>3</sup> Incubation Time—48 to 72 hours minimum Incubation Temperature—30° to 35°
<i>Water for Injection:</i>	Membrane Filtration Method <sup>1</sup>
	Sample Volume—100 mL minimum <sup>2</sup> Growth Medium—Plate Count Agar <sup>3</sup> Incubation Time—48 to 72 hours minimum Incubation Temperature—30°C to 35°C
<sup>1</sup> A membrane filter with a rating of 0.45 µm is generally considered preferable even though the cellular width of some of the bacteria in the sample may be narrower than this. The efficiency of the filtration process still allows the retention of a very high percentage of these smaller cells and is adequate for this application. Filters with smaller ratings may be used if desired, but for a variety of reasons the ability of the retained cells to develop into visible colonies may be compromised, so count accuracy must be verified by a reference approach.	
<sup>2</sup> When colony counts are low to undetectable using the indicated minimum sample volume, it is generally recognized that a larger sample volume should be tested in order to gain better assurance that the resulting colony count is more statistically representative. The sample volume to consider testing is dependent on the user's need to know (which is related to the established alert and action levels and the water system's microbial control capabilities) and the statistical reliability of the resulting colony count. In order to test a larger sample volume, it may be necessary to change testing techniques, e.g., changing from a pour plate to a membrane filtration approach. Nevertheless, in a very low to nil count scenario, a maximum sample volume of around 250 to 300 mL is usually considered a reasonable balance of sample collecting and processing ease and increased statistical reliability. However, when sample volumes larger than about 2 mL are needed, they can only be processed using the membrane filtration method.	
<sup>3</sup> Also known as Standard Methods Agar, Standard Methods Plate Count Agar, or TGYA, this medium contains tryptone (pancreatic digest of casein), glucose and yeast extract.	

## IDENTIFICATION OF MICROORGANISMS

Identifying the isolates recovered from water monitoring methods may be important in instances where specific waterborne microorganisms may be detrimental to the products or processes in which the water is used. Microorganism information such as this may also be useful when identifying the source of microbial contamination in a product or process. Often a limited group of microorganisms is routinely recovered from a water system. After repeated recovery and characterization, an experienced microbiologist may become proficient at their identification based on only a few recognizable traits such as colonial morphology and staining characteristics. This may allow for a reduction in the number of identifications to representative colony types, or, with proper analyst qualification, may even allow testing short cuts to be taken for these microbial

identifications.

## ALERT AND ACTION LEVELS AND SPECIFICATIONS

Though the use of alert and action levels is most often associated with microbial data, they can be associated with any attribute. In pharmaceutical water systems, almost every quality attribute, other than microbial quality, can be very rapidly determined with near-real time results. These short-delay data can give immediate system performance feedback, serving as ongoing process control indicators. However, because some attributes may not continuously be monitored or have a long delay in data availability (like microbial monitoring data), properly established *Alert and Action Levels* can serve as an early warning or indication of a potentially approaching quality shift occurring between or at the next periodic monitoring. In a validated water system, process controls should yield relatively constant and more than adequate values for these monitored attributes such that their *Alert and Action Levels* are infrequently broached.

As process control indicators, alert and action levels are designed to allow remedial action to occur that will prevent a system from deviating completely out of control and producing water unfit for its intended use. This “intended use” minimum quality is sometimes referred to as a “specification” or “limit” . In the opening paragraphs of this chapter, rationale was presented for no microbial specifications being included within the body of the bulk water (*Purified Water* and *Water for Injection*) monographs. This does not mean that the user should not have microbial specifications for these waters. To the contrary, in most situations such specifications should be established by the user. The microbial specification should reflect the maximum microbial level at which the water is still fit for use without compromising the quality needs of the process or product where the water is used. Because water from a given system may have many uses, the most stringent of these uses should be used to establish this specification.

Where appropriate, a microbial specification could be qualitative as well as quantitative. In other words, the number of total microorganisms may be as important as the number of a specific microorganism or even the absence of a specific microorganism.

Microorganisms that are known to be problematic could include opportunistic or overt pathogens, nonpathogenic indicators of potentially undetected pathogens, or microorganisms known to compromise a process or product, such as by being resistant to a preservative or able to proliferate in or degrade a product. These microorganisms comprise an often ill-defined group referred to as “objectionable microorganisms” .

Because objectionable is a term relative to the water's use, the list of microorganisms in such a group should be tailored to those species with the potential to be present and problematic. Their negative impact is most often demonstrated when they are present in high numbers, but depending on the species, an allowable level may exist, below which they may not be considered objectionable.

As stated above, alert and action levels for a given process control attribute are used to help maintain system control and avoid exceeding the pass/fail specification for that attribute. Alert and action levels may be both quantitative and qualitative. They may involve levels of total microbial counts or recoveries of specific microorganisms. Alert levels are events or levels that, when they occur or are exceeded, indicate that a process may have drifted from its normal operating condition. Alert level excursions constitute a warning and do not necessarily require a corrective action. However, alert level excursions usually lead to the alerting of personnel involved in water system operation as well as QA. Alert level excursions may also lead to additional monitoring with more intense scrutiny of resulting and neighboring data as well as other process indicators. Action levels are events or higher levels that, when they occur or are exceeded, indicate that a process is probably drifting from its normal operating range. Examples of kinds of action level “events” include exceeding alert levels repeatedly; or in multiple simultaneous locations, a single occurrence of exceeding a higher microbial level; or the individual or repeated recovery of specific objectionable microorganisms. Exceeding an action level should lead to immediate notification of both QA and personnel involved in water system operations so that corrective actions can immediately be taken to bring the process back into its normal operating range. Such remedial actions should also include efforts to understand and eliminate or at least reduce the incidence of a future occurrence. A root cause investigation may be necessary to devise an effective preventative action strategy. Depending on the nature of the action level excursion, it may also be necessary to evaluate its impact on the water uses during that time. Impact evaluations may include delineation of affected batches and additional or more extensive product testing. It may also involve experimental product challenges.

Alert and action levels should be derived from an evaluation of historic monitoring data called a trend analysis. Other guidelines on approaches that may be used, ranging from “inspectional” to statistical evaluation of the historical data have been published. The ultimate goal is to understand the normal variability of the data during what is considered a typical operational period. Then, trigger points or levels can be established that will signal when future data may be approaching (alert level) or exceeding (action level) the boundaries of that “normal variability”. Such alert and action levels are based on the control capability of the system as it was being maintained and controlled during that historic period of typical control.

In new water systems where there is very limited or no historic data from which to derive data trends, it is common to simply establish initial alert and action levels based on a combination of equipment design capabilities but below the process and product specifications where water is used. It is also common, especially for ambient water systems, to microbiologically “mature” over the first year of use. By the end of this period, a relatively steady state microbial population (microorganism types and levels)



will have been allowed or promoted to develop as a result of the collective effects of routine system maintenance and operation, including the frequency of unit operation rebeddings, backwashings, regenerations, and sanitizations. This microbial population will typically be higher than was seen when the water system was new, so it should be expected that the data trends (and the resulting alert and action levels) will increase over this “maturation” period and eventually level off.

A water system should be designed so that performance-based alert and action levels are well below water specifications. With poorly designed or maintained water systems, the system owner may find that initial new system microbial levels were acceptable for the water uses and specifications, but the mature levels are not. This is a serious situation, which if not correctable with more frequent system maintenance and sanitization, may require expensive water system renovation or even replacement. Therefore, it cannot be overemphasized that water systems should be designed for ease of microbial control, so that when monitored against alert and action levels, and maintained accordingly, the water continuously meets all applicable specifications.

An action level should not be established at a level equivalent to the specification. This leaves no room for remedial system maintenance that could avoid a specification excursion. Exceeding a specification is a far more serious event than an action level excursion. A specification excursion may trigger an extensive finished product impact investigation, substantial remedial actions within the water system that may include a complete shutdown, and possibly even product rejection.

Another scenario to be avoided is the establishment of an arbitrarily high and usually nonperformance based action level. Such unrealistic action levels deprive users of meaningful indicator values that could trigger remedial system maintenance.

Unrealistically high action levels allow systems to grow well out of control before action is taken, when their intent should be to catch a system imbalance before it goes wildly out of control.

Because alert and action levels should be based on actual system performance, and the system performance data are generated by a given test method, it follows that those alert and action levels should be valid only for test results generated by the same test method. It is invalid to apply alert and action level criteria to test results generated by a different test method. The two test methods may not equivalently recover microorganisms from the same water samples. Similarly invalid is the use of trend data to derive alert and action levels for one water system, but applying those alert and action levels to a different water system. Alert and action levels are water system and test method specific.

Nevertheless, there are certain maximum microbial levels above which action levels should never be established. Water systems with these levels should unarguably be considered out of control. Using the microbial enumeration methodologies suggested

above, generally considered maximum action levels are 100 cfu per mL for *Purified Water* and 10 cfu per 100 mL for *Water for Injection*. However, if a given water system controls microorganisms much more tightly than these levels, appropriate alert and action levels should be established from these tighter control levels so that they can truly indicate when water systems may be starting to trend out of control. These in-process microbial control parameters should be established well below the user-defined microbial specifications that delineate the water's fitness for use.

Special consideration is needed for establishing maximum microbial action levels for Drinking Water because the water is often delivered to the facility in a condition over which the user has little control. High microbial levels in Drinking Water may be indicative of a municipal water system upset, broken water main, or inadequate disinfection, and therefore, potential contamination with objectionable microorganisms. Using the suggested microbial enumeration methodology, a reasonable maximum action level for Drinking Water is 500 cfu per mL. Considering the potential concern for objectionable microorganisms raised by such high microbial levels in the feedwater, informing the municipality of the problem so they may begin corrective actions should be an immediate first step. In-house remedial actions may or may not also be needed, but could include performing additional coliform testing on the incoming water and pretreating the water with either additional chlorination or UV light irradiation or filtration or a combination of approaches.

**Auxiliary Information**— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Antonio Hernandez-Cardoso, M.Sc.</a> Senior Scientific Liaison 1-301-816-8308	(GCCA2010) General Chapters - Chemical Analysis

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## 《 2021 》 MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS

### INTRODUCTION

This chapter provides tests for the estimation of the number of viable aerobic microorganisms present in nutritional supplements of all kinds, from raw materials to the finished forms. Alternative methods may be substituted for the tests, provided that they have been properly validated as giving equivalent or better results. In preparing for and in applying the tests, observe aseptic precautions in handling the specimens. The term “growth” is used in a special sense herein, i.e., to designate the presence and presumed proliferation of viable microorganisms.

### PREPARATORY TESTING

The validity of the results of the tests set forth in this chapter rests largely upon the adequacy of a demonstration that the test specimens to which they are applied do not, of themselves, inhibit the multiplication, under the test conditions, of microorganisms that may be present. Therefore, preparatory to conducting the tests on a regular basis and as circumstances require subsequently, inoculate diluted specimens of the material to be tested with separate viable cultures of the challenge microorganisms.

For the *Soybean – Casein Digest Agar* used for *Total Aerobic Microbial Counts*, inoculate duplicate plates with 25 to 250 cfu of *Staphylococcus aureus* (ATCC<sup>1</sup>No. 6538), *Escherichia coli* (ATCC No. 8739), and *Bacillus subtilis* (ATCC No. 6633) to demonstrate a greater than 70% bioburden recovery in comparison to a control medium. For the *Sabouraud Dextrose Agar* used for *Total Combined Yeast and Mold Counts*, inoculate duplicate plates with 25 to 250 cfu of *Candida albicans* (ATCC No. 10231) and *Aspergillus niger* (ATCC No. 16404) to demonstrate a greater than 70% bioburden recovery in comparison to a control medium. For *Enterobacterial Probable Number Determinations (Bile-Tolerant Gram-Negative Bacteria)*, appropriate dilutions of *Escherichia coli* (ATCC No. 8739) and *Salmonella typhimurium* (ATCC No. 13311) are used. Failure of the organism(s) to grow in the relevant medium invalidates that portion of the examination and necessitates a modification of the procedure by (1) an increase in the volume of diluent, the quantity of test material remaining the same, or by (2) the incorporation of a sufficient quantity of suitable inactivating agent(s) in the diluents, or by (3) an appropriate combination of modifications to (1) and (2) so as to permit growth of the inoculum.

The following are examples of ingredients and their concentrations that may be added to the culture medium to neutralize inhibitory substances present in the sample: soy lecithin, 0.5%; and polysorbate 20, 4.0%. Alternatively, repeat the test as described in

the preceding paragraph, using *Fluid Casein Digest – Soy Lecithin – Polysorbate 20 Medium* to demonstrate neutralization of preservatives or other antimicrobial agents in the test material. Where inhibitory substances are contained in the product and the latter is soluble, a suitable, validated adaptation of a procedure set forth under *Procedures* using the *Membrane Filtration Method* may be used.

If, in spite of the incorporation of suitable inactivating agents and a substantial increase in the volume of diluent, it is still not possible to recover the viable cultures described above, and where the article is not suitable for the employment of membrane filtration, it can be assumed that the failure to isolate the inoculated organism is attributable to the bactericidal or bacteriostatic activity of such magnitude that treatments are not able to remove the activity. This information serves to indicate that the article is not likely to allow proliferation or contamination with the given species of microorganism. Monitoring should be continued in order to determine the inhibitory range and bactericidal activity of the article.

## **BUFFER SOLUTION AND MEDIA**

Culture media may be prepared as follows, or dehydrated culture media may be used provided that, when reconstituted as directed by the manufacturer or distributor, they have similar ingredients and/or yield media comparable to those obtained from the formulas given herein.

In preparing media by the formulas set forth herein, dissolve the soluble solids in the water, using heat if necessary to effect complete solution, and add solutions of hydrochloric acid or sodium hydroxide in quantities sufficient to yield the desired pH in the medium when it is ready for use. Determine the pH at  $25 \pm 2^\circ$ .

Where agar is called for in a formula, use agar that has a moisture content of not more than 15%. Where water is called for in a formula, use [Purified Water](#).

### **pH 7.2 Phosphate Buffer**

Prepare a stock solution by dissolving 34 g of monobasic potassium phosphate in about 500 mL of water contained in a 1000-mL volumetric flask. Adjust to a pH of  $7.2 \pm 0.1$  by the addition of sodium hydroxide TS (about 175 mL), add water to volume, and mix. Dispense and sterilize. Store under refrigeration. For use, dilute the stock solution with water in the ratio of 1 to 800, dispense as desired, and sterilize.

### **Media**

Prepare media for the tests as described below. Alternatively, dehydrated formulations may be used provided that, when reconstituted as directed by the manufacturer or distributor, they meet the requirements of the *Growth Promotion Testing*. Unless otherwise indicated elsewhere in this chapter, media are sterilized in autoclaves using a

validated process. The exposure time within the autoclave at 121° will depend on the volume of media to be sterilized. Thus, for example, a 500-mL volume would need to be autoclaved using a temperature and time relationship that will ensure that the medium has attained at least an  $F_0$  of 12 – 15 in the sterilization process. However, the appropriate time and temperature duration for sterilizing prepared media at any given volume should be confirmed by a thermal penetration study using a thermocouple or thermoprobe placed within the liquid medium.

#### FLUID CASEIN DIGEST – SOY LECITHIN – POLYSORBATE 20 MEDIUM

Pancreatic Digest of Casein	20 g
Soy Lecithin	5 g
Polysorbate 20	40 mL
Water	960 mL

Dissolve pancreatic digest of casein and soy lecithin in 960 mL of water, heating in a water bath at 48° to 50° for about 30 minutes to effect solution. Add 40 mL of polysorbate 20. Mix, dispense as desired, and sterilize.

#### SOYBEAN – CASEIN DIGEST – AGAR MEDIUM

Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean Meal	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Water	1000 mL

pH after sterilization:  $7.3 \pm 0.2$ .

#### FLUID SOYBEAN – CASEIN DIGEST MEDIUM

Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean Meal	3.0 g
Sodium Chloride	5.0 g
Dibasic Potassium Phosphate	2.5 g
Dextrose	2.5 g
Purified Water	1000 mL

Dissolve the solids in the water, heating slightly to effect a solution. Cool the solution to room temperature, and adjust the pH with 1 N sodium hydroxide so that after sterilization it will have a pH of  $7.3 \pm 0.2$ . Filter, if necessary, and dispense into suitable containers. Sterilize at a temperature and time relationship that will ensure that the medium has attained at least an  $F_0$  of 12 – 15 in the sterilization process, or by a validated filtration process.

#### SABOURAUD DEXTROSE – AGAR MEDIUM

Dextrose	40.0 g
Mixture of Peptic Digest of Animal Tissue and Pancreatic Digest of Casein	

(1:1)	10.0 g
Agar	15.0 g
Water	1000 mL

Mix, and boil to effect solution.

pH after sterilization:  $5.6 \pm 0.2$ .

#### VIOLET-RED BILE AGAR WITH GLUCOSE AND LACTOSE

Yeast Extract	3.0 g
Pancreatic Digest of Gelatin	7.0 g
Bile Salts	1.5 g
Lactose	10.0 g
Sodium Chloride	5.0 g
D-Glucose Monohydrate	10.0 g
Agar	15.0 g
Neutral Red	30 mg
Crystal Violet	2 mg
Water	1000 mL

Adjust the pH so that it is  $7.4 \pm 0.2$  after heating. Heat to boiling, but do not heat in an autoclave. Pour onto plates.

#### MOSSEL – ENTEROBACTERIACEAE ENRICHMENT BROTH

Pancreatic Digest of Gelatin	10.0 g
D-Glucose Monohydrate	5.0 g
Dehydrated Ox Bile	20.0 g
Monobasic Potassium Phosphate	2.0 g
Dibasic Potassium Phosphate	8.0 g
Brilliant Green	15 mg
Water	1000 mL

Suspend the solids in water, and heat to boiling for 1 to 2 minutes. Transfer 120-mL portions to 250-mL volumetric flasks or 9-mL portions to test tubes, all being capped with cotton plugs or loose-fitting caps. Heat on a steam bath for 30 minutes. Adjust the pH so that it is  $7.2 \pm 0.2$  after heating.

#### GROWTH PROMOTION TESTING

Each lot of dehydrated medium bearing the manufacturer's identifying number or each lot of medium prepared from basic ingredients must be tested for its growth-promoting qualities. Cultures of *Staphylococcus aureus* (ATCC No. 6538), *Escherichia coli* (ATCC No. 8739), *Bacillus subtilis* (ATCC No. 6633), *Candida albicans* (ATCC No. 10231), and *Aspergillus niger* (ATCC No. 16404) are used. A  $10^{-3}$  dilution of a 24-hour broth culture of the microorganism to the first dilution (in pH 7.2 Phosphate Buffer or Fluid Soybean –

*Casein Digest Medium*) may be used as the inocula. Serially streak plates of the media with the appropriate inocula to obtain isolated colonies to demonstrate the growth-promotion qualities of the *Soybean – Casein Digest* and *Sabouraud Dextrose Agar* media. Inoculate the *Fluid Soybean – Casein Digest Medium* and *Mossel – Enterobacteriaceae Enrichment Broth* with 10 to 100 cfu of the appropriate challenge organisms to demonstrate their growth-promotion qualities.

## SAMPLING

Provide 10-mL or 10-g specimens for the tests called for in the individual monograph.

## PROCEDURE

Prepare the specimen to be tested by a treatment that is appropriate to its physical characteristics and that does not alter the number and kind of microorganisms originally present, in order to obtain a solution or suspension of all or part of it in a form suitable for the test procedure(s) to be carried out.

For a solid that dissolves to an appreciable extent but not completely, reduce the substance to a moderately fine powder, suspend it in the vehicle specified, and proceed as directed under *Total Aerobic Microbial Count*.

For a fluid specimen that consists of a true solution, or a suspension in water or a hydroalcoholic vehicle containing less than 30% of alcohol, and for a solid that dissolves readily and practically completely in 90 mL of *pH 7.2 Phosphate Buffer* or the media specified, proceed as directed under *Total Aerobic Microbial Count*.

For water-immiscible products, prepare a suspension with the aid of a minimal quantity of a suitable, sterile emulsifying agent (such as one of the polysorbates), using a mechanical blender and warming to a temperature not exceeding 45°, if necessary, and proceed with the suspension as directed under *Total Aerobic Microbial Count*.

### Total Aerobic Microbial Count

For specimens that are freely soluble, use the *Membrane Filtration Method* or *Plate Method*. For specimens that are sufficiently soluble or translucent to permit use of the *Plate Method*, use that method; otherwise, use the *Multiple-Tube Method*. With either method, first dissolve or suspend 10.0 g of the specimen if it is a solid, or 10 mL, accurately measured, if the specimen is a liquid, in *pH 7.2 Phosphate Buffer*, *Fluid Soybean – Casein Digest Medium*, or *Fluid Casein Digest – Soy Lecithin – Polysorbate 20 Medium* to make 100 mL. For viscous specimens that cannot be pipeted at this initial 1:10 dilution, dilute the specimen until a suspension is obtained, i.e., 1:50 or 1:100, etc., that can be pipeted. Perform the test for absence of inhibitory (antimicrobial) properties as described under *Preparatory Testing* before the determination of *Total Aerobic*

**Microbial Count.** Add the specimen to the medium not more than 1 hour after preparing the appropriate dilutions for inoculation.

### **Membrane Filtration Method**

Dilute the fluid further, if necessary, so that 1 mL will be expected to yield between 30 and 300 colonies. Pipet 1 mL of the final dilution into 5 to 10 mL of *pH 7.2 Phosphate Buffer*, *Fluid Soybean – Casein Digest Medium*, or *Fluid Casein Digest – Soy Lecithin – Polysorbate 20 Medium*. Wash each membrane with an appropriate amount of one of the above diluents. Transfer each membrane to a Petri dish containing *Soybean – Casein Digest Agar Medium*, previously solidified at room temperature. Incubate the plates at a temperature between 30° and 35° for 48 to 72 hours. Following incubation, examine the plates for growth, count the number of colonies, and express the average for the two plates in terms of the number of microorganisms per g or per mL of specimen. If no microbial colonies are recovered from the dishes representing the initial 1:10 dilution of the specimen, express the results as “less than 10 microorganisms per g or per mL of specimen.”

### **Plate Method**

Dilute the fluid further, if necessary, so that 1 mL will be expected to yield between 30 and 300 colonies. Pipet 1 mL of the final dilution onto each of two sterile Petri dishes. Promptly add to each dish 15 to 20 mL of *Soybean – Casein Digest – Agar Medium*, previously melted and cooled to about 45°. Cover the Petri dishes, mix the sample with agar by gently tilting or rotating the dishes, and allow the contents to solidify at room temperature. Invert the Petri dishes and incubate for 48 to 72 hours. Following incubation, examine the plates for growth, count the number of colonies, and express the average for the two plates in terms of the number of microorganisms per g or per mL of specimen. If no microbial colonies are recovered from the dishes representing the initial 1:10 dilution of the specimen, express the results as “less than 10 microorganisms per g or per mL of specimen.”

### **Multiple-Tube Method**

Into each of 14 test tubes of similar size, place 9.0 mL of sterile *Fluid Soybean – Casein Digest Medium*. Arrange 12 of the tubes in four sets of three tubes each. Put aside one set of three tubes to serve as the controls. Into each of three tubes of one set ( “100” ) and into a fourth tube (A) pipet 1 mL of the solution or suspension of the specimen, and mix. Pipet 1 mL from tube A into the one remaining tube (B), not included in a set, and mix. These two tubes contain 100 mg or 100 µL and 10 mg or 10 µL of the specimen, respectively. Into each of the second set ( “10” ) of three tubes pipet 1 mL from tube A, and into each tube of the third set ( “1” ) pipet 1 mL from tube B. Discard the unused contents of tubes A and B. Close well, and incubate all of the tubes. Following incubation, examine the tubes for growth: the three control tubes remain clear, and the



observations in the tubes containing the specimen, when interpreted by reference to [Table 1](#), indicate the most probable number of microorganisms per g or per mL.

**Table 1. Most Probable Count by Multiple-Tube Method**

Observed Combinations of Numbers of Tubes Showing Growth in Each Set			Most Probable Number of Microorganisms per g or per mL
Number of mg or µL of specimen per tube			
100	10	1	
3	3	3	more than 1100
3	3	2	1100
3	3	1	500
3	3	0	200
3	2	3	290
3	2	2	210
3	2	1	150
3	2	0	90
3	1	3	160
3	1	2	120
3	1	1	70
3	1	0	40
3	0	3	95
3	0	2	60
3	0	1	40
3	0	0	23
2	2	0	21
2	1	1	20
2	1	0	15
2	0	1	14
2	0	0	9
1	2	0	11
1	1	0	7
1	0	0	4
0	1	0	3
0	0	0	<3

#### Total Combined Molds and Yeasts Count

**Procedure**— Proceed as directed for *Membrane Filtration Method* or *Plate Method* under *Total Aerobic Microbial Count*, except to use the same amount of *Sabouraud Dextrose - Agar Medium* instead of *Soybean - Casein Digest - Agar Medium* and to incubate the plates for 5 to 7 days at 20° to 25°.

**Retest**— For the purpose of confirming a doubtful result by any of the procedures outlined in the foregoing tests following their application to a 10-g specimen, a retest on an additional 10-g specimen from the original sample and a 10-g specimen from the new sample of the nutritional supplement may be conducted. Proceed as directed under *Procedure*.

### Enterobacterial Count (Bile-Tolerant Gram-Negative Bacteria)

Dissolve or suspend the sample in a sufficient volume of *pH 7.2 Phosphate Buffer* or *Fluid Soybean - Casein - Digest Medium* and dilute with *Fluid Soybean - Casein - Digest Medium* to 100 mL. Pre-incubate for 2 to 5 hours at 20° - 25° in *Soybean - Casein Digest Broth* diluent; inoculate suitable quantities of *Mossel - Enterobacteriaceae Enrichment Broth* to contain 0.1, 0.01, or 0.001 g or mL of the product. Incubate at 30° - 35° for 24 to 48 hours. Subculture onto a plate of *Violet-Red Bile Agar* with *Glucose* and *Lactose*, and incubate at 30° - 35° for 18 to 24 hours. Growth of well developed, generally red or reddish, colonies of Gram-Negative bacteria reveal the presence of enterobacteria. Determine the most probable number of microorganisms per g or per mL by reference to [Table 2](#).

**Table 2. The Most Probable Enterobacterial Count**

Observed Presence of Enterobacteria			Most Probable Number of Enterobacteria per g
Number of g of specimen per tube			
0.1	0.01	0.001	
+	+	+	more than 100
+	+	–	fewer than 100 but more than 10
+	–	–	fewer than 10 but more than 1
–	–	–	fewer than 1

<sup>1</sup> Available from ATCC, 10801 University Boulevard, Manassas, VA 20110-2209. Equivalent microorganisms, provided that they are from a national collection repository, can be used in lieu of ATCC strains. However, the viable microorganisms used in the test must not be more than five passages removed from the original ATCC or national collection culture.

**Auxiliary Information**— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Principal Scientific Liaison 1-301-816-8339	(GCM2010) General Chapters - Microbiology

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## 《 2022 》 MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS

### INTRODUCTION

Good manufacturing practices require that objectionable organisms be absent from nonsterile nutritional and dietary products. A microorganism can be considered objectionable if it represents a potential health hazard to the user who is using the product as directed, or if it is capable of growing in the product. Objectionable microorganisms are defined as contaminants that, depending on the microbial species, number of organisms, dosage form, intended use, and patient population, would adversely affect product safety. Additionally, microorganisms may be deemed objectionable if they adversely affect product stability or if they may damage the integrity of the container closure system.

This chapter describes the testing of nutritional and dietary articles for specified microorganisms, which are specified in the individual monographs or whose absence is recommended by the guidance under [Microbiological Attributes of Nonsterile Nutritional and Dietary Supplements](#) 《 2023 》. When objectionable microorganisms are not specified in the individual monograph, it is the manufacturers' responsibility to determine which microorganisms in their products are objectionable. It is not intended that all nonsterile nutritional and dietary articles be tested for the absence of all of the microorganisms mentioned in this chapter, nor is the testing of relevant microorganisms restricted to those presented in this chapter.

Alternative microbiological, physicochemical, and biotechnological methods, including automated methods, may be substituted for these tests, provided they have been validated as being equivalent in their suitability for determining compliance.

### BUFFER AND MEDIA

#### General Considerations

See *Buffer Solution and Media* under [Microbial Enumeration Tests—Nutritional and Dietary Supplements](#) 《 2021 》. The appropriateness of each medium for the intended purpose is to be assessed. Control sets of *Fluid Soybean – Casein Digest Medium* for *Preparatory Testing* are also used to assess the appropriateness of these media in the growth promotion of the specified microorganisms. For other media, streak agar plates to obtain isolated colonies of appropriate microorganisms, and inoculate the fluid media with the appropriate microorganisms at a final concentration of less than 100 cfu per mL. Observe the growth to establish the appropriateness of the media.

#### Buffer

**Buffer Stock Solution and pH 7.2 Phosphate Buffer**— Proceed as directed under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* { 2021 }.

### Media

#### FLUID SOYBEAN – CASEIN DIGEST MEDIUM

Prepare as directed under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* { 2021 }.

#### MANNITOL – SALT – AGAR MEDIUM

Pancreatic Digest of Casein	5.0 g
Peptic Digest of Animal Tissue	5.0 g
Beef Extract	1.0 g
D-Mannitol	10.0 g
Sodium Chloride	75.0 g
Agar	15.0 g
Phenol Red	0.025 g
Water	1000 mL

Mix, then heat with frequent agitation, and boil for 1 minute to effect solution.

pH after sterilization:  $7.4 \pm 0.2$ .

#### FLUID TETRATHIONATE MEDIUM

Pancreatic Digest of Casein	2.5 g
Peptic Digest of Animal Fat	2.5 g
Bile Salts	1.0 g
Calcium Carbonate	10.0 g
Sodium Thiosulfate	30.0 g
Water	1000 mL

Heat to boiling. Do not autoclave; use the same day. Immediately before use, add a solution prepared by dissolving 5 g of potassium iodide and 6 g of iodine in 20 mL of water. Then add 10 mL of a solution of brilliant green (1 in 1000), and mix. Do not heat after adding the brilliant green solution.

#### BRILLIANT GREEN – AGAR MEDIUM

Yeast Extract	3.0 g
Peptic Digest of Animal Tissue	5.0 g
Pancreatic Digest of Casein	5.0 g
Lactose	10.0 g
Sodium Chloride	5.0 g
Sucrose	10.0 g
Phenol Red	80.0 g
Agar	20.0 g

Brilliant Green	12.5 mg
Water	1000 mL

Boil for 1 minute. Sterilize just prior to use, melt, pour into Petri dishes, and allow to cool.

pH after sterilization:  $6.9 \pm 0.2$

#### XYLOSE – LYSINE – DESOXYCHOLATE – AGAR MEDIUM

Xylose	3.5 g
L-Lysine	5.0 g
Lactose	7.5 g
Sucrose	7.5 g
Sodium Chloride	5.0 g
Yeast Extract	3.0 g
Phenol Red	80 mg
Agar	13.5 g
Sodium Desoxycholate (as Bile Salts)	2.5 g
Sodium Thiosulfate	6.8 g
Ferric Ammonium Citrate	800 mg
Water	1000 mL

Heat, with swirling, just to the boiling point. Do not overheat or sterilize. Transfer at once to a water bath maintained at about  $50^{\circ}$ , and pour into Petri plates as soon as the *Medium* has cooled.

Final pH:  $7.4 \pm 0.2$ .

#### HEKTOEN ENTERIC AGAR MEDIUM

Protease Peptone	12.0 g
Yeast Extract	3.0 g
Lactose	12.0 g
Sucrose	2.0 g
Salicin	9.0 g
Bile Salts No. 3	9.0 g
Sodium Chloride	5.0 g
Sodium Thiosulfate	5.0 g
Ferric Ammonium Citrate	1.5 g
Acid Fuchsin	0.1 g
Bromothymol Blue	65 mg
Agar	14.0 g
Water	1000 mL

Mix, and allow to stand for 10 minutes. Heat gently, and allow to boil for a few seconds to dissolve the agar. Do not sterilize. Cool to  $60^{\circ}$ , and pour into Petri dishes.

Final pH:  $7.5 \pm 0.2$ .

## TRIPLE SUGAR – IRON – AGAR MEDIUM

Pancreatic Digest of Casein	10.0 g
Pancreatic Digest of Animal Tissue	10.0 g
Lactose	10.0 g
Sucrose	10.0 g
Dextrose	1.0 g
Ferrous Ammonium Sulfate	200 mg
Sodium Chloride	5.0 g
Sodium Thiosulfate	200 mg
Agar	13.0 g
Phenol Red	25 mg
Water	1000 mL

pH after sterilization:  $7.3 \pm 0.2$ .

## MACCONKEY AGAR MEDIUM

Pancreatic Digest of Gelatin	17.0 g
Pancreatic Digest of Casein	1.5 g
Peptic Digest of Animal Tissue	1.5 g
Lactose	10.0 g
Bile Salts Mixture	1.5 g
Sodium Salts Mixture	5.0 g
Agar	13.5 g
Neutral Red	30 mg
Crystal Violet	1.0 mg
Water	1000 mL

Boil for 1 minute to effect solution.

pH after sterilization:  $7.1 \pm 0.2$ .

## LEVINE EOSIN – METHYLENE BLUE – AGAR MEDIUM

Pancreatic Digest of Gelatin	10.0 g
Dibasic Potassium Phosphate	2.0 g
Agar	15.0 g
Lactose	10.0 g
Eosin Y	400 mg
Methylene Blue	65 mg
Water	1000 mL

Dissolve pancreatic digest of gelatin, dibasic potassium phosphate, and agar in water, with warming, and allow to cool. Just prior to use, liquefy the gelled agar solution, and add the remaining ingredients, as solutions, in the following amounts: for each 100 mL of the liquefied agar solution, add 5 mL of lactose solution (1 in 5), 2 mL of the eosin Y

solution (1 in 50), and 2 mL of methylene blue solution (1 in 300). Mix. The finished *Medium* may not be clear.

pH after sterilization:  $7.1 \pm 0.2$ .

#### BAIRD – PARKER AGAR MEDIUM

Pancreatic Digest of Casein	10.0 g
Beef Extract	5.0 g
Yeast Extract	1.0 g
Lithium Chloride	5.0 g
Agar	20.0 g
Glycine	12.0 g
Sodium Pyruvate	10.0 g
Water	950 mL

Heat with frequent agitation, and boil for 1 minute. Sterilize, cool to between  $45^{\circ}$  and  $50^{\circ}$ , and add 10 mL of sterile potassium tellurite solution (1 in 100) and 50 mL of egg yolk emulsion prepared as follows. Disinfect the surface of whole-shell eggs, aseptically crack the eggs, transfer intact yolks to a sterile graduated cylinder, add sterile saline TS to obtain a 3 to 7 ratio of egg yolk to saline, add to a sterile blender cup, and mix at high speed for 5 seconds. Mix all ingredients well but gently, and pour into plates.

pH after sterilization:  $6.8 \pm 0.2$ .

#### VOGEL – JOHNSON AGAR MEDIUM

Pancreatic Digest of Casein	10.0 g
Yeast Extract	5.0 g
Mannitol	10.0 g
Dibasic Potassium Phosphate	5.0 g
Lithium Chloride	5.0 g
Glycine	10.0 g
Agar	16.0 g
Phenol Red	25.0 mg
Water	1000 mL

Boil for 1 minute. Sterilize, cool to between  $45^{\circ}$  and  $50^{\circ}$ , and add 20 mL of sterile potassium tellurite solution (1 in 100).

pH after sterilization:  $7.2 \pm 0.2$ .

#### FLUID SELENITE – CYSTINE MEDIUM

Pancreatic Digest of Casein	5.0 g
Lactose	4.0 g
Sodium Phosphate	10.0 g
Sodium Acid Selenite	4.0 g
L-Cystine	10.0 g

Water	1000 mL
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Mix, and heat to effect solution. Then heat in flowing stream for 15 minutes. Do not sterilize.

Final pH:  $7.0 \pm 0.2$ .

#### REINFORCED MEDIUM FOR CLOSTRIDIA

Beef Extract	10.0 g
Peptone	10.0 g
Yeast Extract	3.0 g
Soluble Starch	1.0 g
Glucose Monohydrate	5.0 g
Cysteine Hydrochloride	0.5 g
Sodium Chloride	5.0 g
Sodium Acetate	3.0 g
Agar	0.5 g
Water	1000 mL

Dissolve agar in water by heating to boiling, while stirring continuously. Adjust the pH if necessary, and sterilize.

pH after sterilization:  $6.8 \pm 0.2$ .

#### COLUMBIA AGAR

Pancreatic Digest of Casein	10.0 g
Meat Peptic Digest	5.0 g
Heart Pancreatic Digest	3.0 g
Yeast Extract	5.0 g
Cornstarch	1.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Water	1000 mL

Dissolve agar in water by heating to boiling and with continuous stirring. If necessary, adjust the pH. Sterilize, and allow to cool to  $45^{\circ}$  to  $50^{\circ}$ . Add, when necessary, gentamicin sulfate, equivalent to about 20 mg of gentamicin base, and pour into Petri dishes.

Pre-reduction of the medium is recommended.

pH after sterilization:  $7.3 \pm 0.2$ .

#### RAPPAPORT VASSILIADIS SALMONELLA ENRICHMENT BROTH

Soya Peptone	4.5 g
Magnesium Chloride Hexahydrate	29.0 g
Sodium Chloride	8.0 g
Dipotassium Phosphate	0.4 g



Potassium Dihydrogen Phosphate	0.6 g
Malachite Green	0.036 g
Purified Water	1000 mL

Dissolve, warming slightly. Sterilize in an autoclave using a validated cycle, at a temperature not exceeding 115°.

The pH is 5.2 ± 0.2 at 25° after heating and autoclaving.

## PREPARATORY TESTING

Proceed as directed for *Preparatory Testing* under [Microbial Enumeration Tests—Nutritional and Dietary Supplements](#) (2021).

For enrichment broth, selective media, and differential media use an inoculating loop to transfer the inoculum of each test organism to the plated or liquid media being tested. If a plated medium is being tested, streak the surface of plate with the loop in four directions to obtain a pattern of isolated colonies. Incubate the media, and examine the plated or liquid media for the characteristic growth of the inocula (See [Tables 1, 2, 3, and 4](#)).

## SAMPLING

Proceed as directed for *Sampling* under [Microbial Enumeration Tests—Nutritional and Dietary Supplements](#) (2021).

## TEST PROCEDURES

**Test Preparation**— Prepare as directed for *Sampling*. Transfer to a suitable container with 100 mL of *Fluid Soybean - Casein Digest Medium (FSCD)*. Mix by shaking gently. [NOTE—On the basis of results for *Preparatory Testing*, modify the *Test Preparation* as appropriate. ]

### Test for Absence of *Staphylococcus aureus*

Incubate at 30° to 35° for 18 to 24 hours. Streak a loopful from *FSCD* onto the surface of one or more of the following media: *Vogel - Johnson Agar Medium (VJ Agar)*, *Mannitol - Salt - Agar Medium (MS-Agar)*, and *Baird-Parker Agar Medium (BP Agar)*.

Cover the Petri plates, invert them, and incubate at 30° to 35° for 24 to 48 hours.

Examine the plates of *VJ Agar*, *MS-Agar*, and/or *BP Agar*, and interpret the results with reference to [Table 1](#): if no plate contains colonies having the characteristics described, the test specimen meets the requirement for the absence of *Staphylococcus aureus*. If characteristic colonies are present, perform coagulase test as follows. Transfer representative colonies to separate tubes containing 0.5 mL of rabbit plasma, horse plasma, or any other mammalian plasma. Incubate in a water bath at 37°. Examine for

coagulation after 3 hours of incubation and at suitable intervals up to 24 hours. Comparing with positive and negative controls, the absence of a coagulase reaction indicates the absence of *Staphylococcus aureus* in the tested article.

**Table 1. Characteristics of *Staphylococcus aureus* on Specified Agar Media**

Agar Medium	Colonial Morphology	Gram Stain
<i>Vogel – Johnson</i>	Black surrounded by yellow zone	(+), cocci
<i>Mannitol – Salt</i>	Yellow colonies with yellow zone	(+), cocci
<i>Baird – Parker</i>	Black, shiny surrounded by 2 – 5-mm clear zones	(+), cocci

### **Test for Absence of *Salmonella* Species**

Incubate at 30° to 35° for 18 to 24 hours. From *FSCD*, pipet a 1-mL aliquot into 10 mL of *Rappaport Vassiliadis Salmonella Enrichment Broth*, mix, and incubate at 30° to 35° for 18 to 24 hours. Streak a loopful from both incubated media onto individual surfaces of one or more of following media: *Brilliant Green Agar Medium (BG-Agar)*, *Xylose – Lysine – Desoxycholate – Agar Medium (XLDC-Agar)*, and *Hektoen Enteric Agar Medium (HE Agar)*. Cover, invert the plates, and incubate at 30° to 35° for 24 to 48 hours. Examine the inoculated plates of *BG-Agar*, *XLDC-Agar*, and/or *HE Agar*, and interpret the results with reference to [Table 2](#): if no colonies having the characteristics described are observed, the test specimen meets the requirement for the absence of *Salmonella* species. If colonies with characteristics described in [Table 2](#) are present, the suspect colonies are transferred to a slant of *Triple Sugar – Iron – Agar Medium (TSI)* using an inoculating wire, by first streaking the surface of the slant, and then stabbing the wire well beneath the surface. Incubate at 30° to 35° for 24 to 48 hours. If the tubes do not have red alkaline slants and yellow acid butts, with or without concomitant blackening of the butts from hydrogen sulfide production, the test specimen meets the requirement for the absence of *Salmonella* species.

**Table 2. Characteristics of *Salmonella* Species on Specified Agar Media**

Agar Medium	Colonial Morphology	Gram Stain
<i>Brilliant Green</i>	Small, transparent and colorless; or opaque, pink or white (often surrounded by pink to red zone)	( – ), rods
<i>Xylose – Lysine – Desoxycholate</i>	Red, with or without black centers	( – ), rods
<i>Hektoen Enteric</i>	Blue-green, with or without black centers	( – ), rods

### **Test for Absence of *Escherichia coli***

Incubate at 30° to 35° for 24 to 48 hours. From *FSCD*, pipet a 1-mL aliquot into a container containing 10 mL of *MacConkey Broth*, mix, and incubate at 42° to 44° for 24 to 48 hours. Streak a loopful from both incubated media onto individual surfaces of

*MacConkey Agar Medium (MC Agar)*, and incubate at 30° to 35° for 18 to 24 hours.

Examine the inoculated *MC Agar* plate, and interpret the results with reference to [Table 3](#): if no colonies having the characteristics described are observed, the test specimen meets the requirement for the absence of *Escherichia coli*. Suspect colonies showing the characteristics described in [Table 3](#) are transferred individually, using an inoculating loop, to the surface of a plate with *Levine Eosin – Methylene Blue – Agar Medium (LEMB-Agar)*. If a large number of suspect colonies are to be transferred, divide the surface of each plate into quadrants, each quadrant being inoculated with a different colony. Cover the plates, invert, and incubate at 30° to 35° for 24 to 48 hours. If none of the colonies exhibit a characteristic metallic sheen under reflected light, and if none exhibit a blue-black appearance under transmitted light, the test specimen meets the requirement for the absence of *Escherichia coli*.

**Table 3. Characteristics of *Escherichia coli* on *MacConkey Agar Medium***

Colonial Morphology	Gram Stain
Brick red, may have surrounding zone of precipitated bile	(–), rods

### Test for Absence of *Clostridium* Species

**Test Preparation**— Prepare as directed for *Sampling*. [NOTE—On the basis of results for *Preparatory Testing*, modify the *Test Preparation* as appropriate. ]

**Procedure**— Take two equal portions of the *Test Preparation*, heat one to 80° for 10 minutes, and cool rapidly. Transfer 10 mL of each portion to separate containers, each containing 100 mL of *Reinforced Medium for Clostridia*, and incubate under anaerobic conditions at 35° to 37° for 48 hours. After incubation, subculture each specimen on *Columbia Agar Medium* to which gentamicin has been added, and incubate under anaerobic conditions at 35° to 37° for 48 hours. Examine the plates, and interpret with reference to [Table 4](#): if no growth of microorganisms is detected, the test specimen meets the requirement for the absence of *Clostridium* species.

**Table 4. Characteristics of *Clostridium* Species on Specified Media**

Medium	Gram Stain	Catalase
<i>Reinforced Medium for Clostridia</i>	(+), rods	
<i>Columbia Agar</i>	(+), rods	Negative

If growth occurs, subculture each distinct colony on *Columbia Agar Medium*, and separately incubate in aerobic and in anaerobic conditions at 35° to 37° for 48 hours. The occurrence of only anaerobic growth of gram-positive bacilli, giving a negative catalase reaction, indicates the presence of *Clostridium sporogenes*. To perform the catalase test, transfer discrete colonies to glass slides, and apply a drop of dilute hydrogen peroxide solution: the reaction is negative if no gas bubbles evolve. If the test specimen exhibits none of these characteristics, it meets the requirement for the absence of *Clostridium* species.

### Retest

For the purpose of confirming a doubtful result by any of the procedures outlined in the foregoing tests following their application to a 10 g specimen, a retest on a 25 g specimen of the nutritional or dietary supplement may be conducted. Proceed as directed under *Procedure*, but make allowances for the larger specimen size.

**Auxiliary Information**— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Principal Scientific Liaison 1-301-816-8339	(GCM2010) General Chapters - Microbiology

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## **《 2023 》 MICROBIOLOGICAL ATTRIBUTES OF NONSTERILE NUTRITIONAL AND DIETARY SUPPLEMENTS**

The raw materials, pharmaceutical ingredients, and active ingredients used in the manufacture of nutritional and dietary articles may range from chemically synthesized vitamins to plant extracts and animal byproducts, and these ingredients are typically not sterile. Considerable experience has accrued with these highly refined plant- and animal-derived pharmaceutical ingredients, such as microcrystalline cellulose, modified starch, lactose, and magnesium stearate, and their microbiological attributes are well established. Botanicals may be microbiologically contaminated at any point during cultivation, harvesting, processing, packing, and distribution. Major sources of microbial contamination are associated with human or animal feces used as plant manure, contaminated irrigation water and/or process water, and poor worker hygiene and sanitation practices during harvesting, sorting, processing, packaging, and transportation. Furthermore, it is essential that microbiological contamination be minimized during the manufacture of nonsterile dietary supplements. To achieve this, Good Manufacturing Practices are employed and adequate microbiological specifications are established.

Microbiological process control, control of the bioburden of raw materials, and control of the manufacturing process to minimize cross-contamination are necessary to guarantee acceptable microbial quality in the final dosage forms. Because nonaqueous or dry dosage forms do not support microbial growth because of low water activity, the microbial quality of such articles is a function of the microorganisms introduced through ingredients or during processing. In addition to considering the intended use of the product, the frequency of microbial testing for the finished nonsterile dietary supplement would be a function of the historical microbial testing database of that product, knowledge of the manufacturing processes, the susceptibility of the formulation to microbial proliferation, and the demonstrated effectiveness of programs controlling the raw materials.

### **FORMULATION AND PROCESS DESIGN**

From a microbiological perspective, the development of the formulation of nutritional or dietary supplements includes an evaluation of raw materials and their suppliers and the contribution made to the products by each ingredient and the manufacturing processes. Characterization of these elements allows the adequacy of the manufacturing process to be demonstrated. For example, if a product is formulated with an ingredient of botanical or animal origin known to possess a high, variable, or unpredictable level of microbiological contamination, it is necessary to ensure that the microbiological monitoring identifies ingredients that have an inappropriate bioburden level and that a premanufacturing process such as drying, extraction, heat treatment, irradiation, or

gaseous sterilization treatment will inactivate or remove any objectionable contaminant possibly present.

However, the selected treatment technique should not have any adverse effects. The treatment of raw materials by irradiation and ethylene oxide may cause unwanted changes affecting the safety and efficacy of the raw material. For instance, when treated by ethylene oxide, crude extracts containing alkaloids have shown reduced contents of alkaloids. Dry heat treatment has been used for inactivation as well, but it requires further evaluation because it may adversely affect stability and degradation of the raw material. With regard to the design of the manufacturing process, appropriate consideration should be given to the microbiological effect of wet granulation manufacturing processes. Wetting of a dry powder can result in increased levels of microorganisms if the granulation is stored prior to drying. However, it is recognized that the pressure and temperature associated with compression of tablets will decrease microbial counts. Antimicrobial activity is also achieved, especially with aqueous preparations, by the addition of chemicals that have known antimicrobial properties and that are compatible with the formulation.

However, antimicrobial preservation is not a substitute for Good Manufacturing Practices. A process has to be designed to minimize the microbiological population. Operating procedures and temperatures and time limits, including holding times, are established to protect the product from microbiological contamination and growth. All processes have to be validated for their intended purposes. Moreover, in-process manufacturing and testing controls necessary for microbiological quality should be identified and implemented.

## **FACILITIES, EQUIPMENT, WATER, AND SANITIZATION**

**Facilities**— The facilities, including the building and the heating, ventilation, and air-conditioning (HVAC) systems, should be designed to minimize microbiological contamination. The design of facilities used for the manufacture of supplements and their operating parameters should be documented, and the documentation should include, when appropriate, HVAC filter types, space pressure differentials, temperature, and relative humidity and air changes. Dry products processed in a dry environment do not possess a high potential for increased microbial levels. However, some control is warranted to minimize microbiological and chemical contamination. Potentially problematic areas are those that utilize [Purified Water](#) for wet granulation, batching liquid products, and film-coating tablets, because water encourages microbial growth.

**Equipment**— Equipment used for the processing of semisolid and dry supplements should be designed to promote sanitary conditions, to be self-drying, and to be easy to clean. Dryers, ovens, wet granulation equipment, bulk tanks, and equipment for

preparation of coating solutions are periodically evaluated to ensure that cleaning procedures are adequate.

**Water**— As one of the major components in nutritional and dietary supplement manufacturing processes, water deserves a special consideration in the microbiological control of these articles. It is a growth medium for a variety of microorganisms that present a threat to product quality, safety, preservation, and stability. Water may even act as a carrier of objectionable microorganisms. In view of this, water used in manufacturing is [Purified Water](#). For the manufacture of raw materials, process water that meets specific microbiological objectives and U.S. Environmental Protection Agency National Drinking Water standards or equivalent European and Japanese standards may be used.

**Cleaning and Sanitization**— Detailed and specific cleaning and sanitization procedures should be evaluated, developed, and validated, with special attention given to product contact surfaces. Personnel should possess sufficient knowledge of these procedures.

## SUPPLEMENT COMPONENTS

Raw materials, excipients, and active substances as components of nutritional and dietary supplements can be a primary source of microbiological contamination. Specifications should be developed and sampling plans and test procedures should be employed to guarantee the desired microbiological attributes of these materials. The nature and extent of microbiological testing should be based upon a knowledge of the material's origin, its manufacturing process, its use, and historical data and experience. For instance, materials of animal or botanical origin that are not highly refined might require special, more frequent testing than synthetic products.

Since members of the family *Enterobacteriaceae* are a major component of the normal epiphytic and endophytic microflora (e.g., members of genera *Klebsiella*, *Enterobacter*, and *Erwinia*) and have been associated with the seeds, pods, roots, leaves, and stems of plants of economic importance, *Coliform* or *Enterobacteriaceae* counts will not be an appropriate general microbiological criterion for botanicals. However, when it is considered advantageous, *Coliform* or *Enterobacteriaceae* counts may be included in the individual monographs. Typically on new leaves, bacteria predominate in the microflora, while yeast and filamentous fungi succeed bacteria and become dominant late in the growing season. With dried botanicals, the bacterial population will tend to change from Gram-negative bacteria to Gram-positive spore formers and fungi. Refinement of botanicals from chopped or powdered plant material to powdered extracts using alcoholic, alkaline, acid hydro-alcoholic, or aqueous extracting materials will reduce the likelihood of vegetative microorganisms within the botanical material. The classification of botanical materials is contained in [Table 1](#).

**Table 1. Definitions of a Range of Botanical Materials**



<b>Botanical Preparation</b>	<b>Definition</b>
Chopped or Powdered Botanicals	Hand-picked portions of the botanical (e.g., leaves, flowers, roots, tubers, etc.) that are air dried, and chopped, flaked, sectioned, ground, or pulverized to the consistency of a powder.
Botanical Extracts	Extracts are solids or semisolid preparations of a botanical that are prepared by percolation, filtration, and concentration by evaporation of the percolate. The extracting material may be alcoholic, alkaline, acid hydro-alcoholic, or aqueous in nature. Typically an extract is 4 to 10 times as strong as the original botanical. The extracts may be semisolids or dry powders termed powdered extracts.
Tinctures	Tinctures are solutions of botanical substances in alcohol obtained by extraction of the powdered, flaked, or sectioned botanical.
Infusions	Infusions are solutions of botanical principles obtained by soaking the powdered botanical in hot or cold water for a specified time and straining. Typically infusions are 5% in strength.
Decoctions	Decoctions are solutions of botanicals prepared by boiling the material in water for at least 15 minutes and straining. Typically decoctions are 5% in strength.
Fluidextracts	A fluidextract is an alcoholic liquid extract made by percolation of a botanical so that 1 mL of the fluidextract represents 1 g of the botanical.
Botanicals to be treated with boiling water before use	Dried botanicals to which boiling water is added immediately prior to consumption.

## **MICROBIOLOGICAL TESTING**

### **Frequency of Sampling and Testing**

Microbiological attribute sampling and testing plans vary widely. In some cases no sampling or testing is necessary; in other cases periodic monitoring is warranted; and yet for some articles each batch requires sampling and testing. The design of the sampling and testing plans and the kind of attributes examined depend on the application and the type of the product, the potential for contamination from components and processing, the growth promotion or inhibition properties of the formulation, and the target population for the supplement. For example, a powdered botanical may have highly variable microbiological attributes so that an incoming batch would be sampled and composite testing would not be advised, while a highly refined botanical extract may not require routine microbial testing. Similarly, products with a low water activity will not be susceptible to microbial growth during their shelf life provided they are protected from elevated humidity by their containers.

### **Microbial Enumeration Tests**

See the *Introduction* under [\*Microbial Enumeration Tests—Nutritional and Dietary\*](#)



[Supplements](#) (2021). These tests provide meaningful information regarding the microbiological acceptability of excipients, active substances, and nonsterile supplement formulations. If the individual monograph does not specify microbial enumeration limits, the guidance provided in this chapter is used. Acceptable general limits of microbial levels for raw materials, excipients, and botanical products are shown in [Table 2](#); and those for raw materials, excipients, active ingredients, and other nonsterile finished articles that are nutritional supplements but do not contain botanicals are shown in [Table 3](#).

**Table 2. Recommended Microbial Limits for Botanical Ingredients and Products**

Material	Recommended Microbial Limit Requirements (cfu/g or mL)
Dried or Powdered Botanicals	Total Aerobic Microbial Count NMT $10^5$
	Total Combined Yeast & Mold Count NMT $10^3$
	Bile-tolerant Gram-negative Bacteria NMT $10^3$
	Absence of <i>Salmonella</i> spp. & <i>E. coli</i> in 10 g
Powdered Botanical Extracts	Total Aerobic Microbial Count NMT $10^4$
	Total Combined Yeast & Mold Count NMT $10^3$
	Absence of <i>Salmonella</i> spp. & <i>E. coli</i> in 10 g
Tinctures	Total Aerobic Microbial Count NMT $10^4$
	Total Combined Yeast & Mold Count NMT $10^3$
Fluidextracts	Total Aerobic Microbial Count NMT $10^4$
	Total Combined Yeast & Mold Count NMT $10^3$
Infusions/Decoctions	Total Aerobic Microbial Count NMT $10^2$
	Total Combined Yeast & Mold Count NMT 10
Nutritional Supplements with Botanicals	Total Aerobic Microbial Count NMT $10^4$
	Total Combined Yeast & Mold Count NMT $10^3$
	Absence of <i>Salmonella</i> spp & <i>E. coli</i> in 10 g
Botanicals to be treated with boiling water before use	Total Aerobic Microbial Count NMT $10^5$

	Total Combined Yeast & Mold Count NMT $10^3$
	Absence of <i>E. coli</i> in 10 g

**Table 3. Recommended Microbial Limits for Dietary Supplement Ingredients and Products**

Material	Recommended Microbial Limit Requirements (cfu/g or mL)
Other raw materials and dietary supplement ingredients	Total Aerobic Microbial Count NMT $10^3$
	Total Combined Yeast & Mold Count NMT $10^2$
	Absence of <i>E. coli</i> in 10 g
Nutritional supplements with synthetic or highly refined ingredients	Total Aerobic Microbial Count NMT $10^3$
	Total Combined Yeast & Mold Count NMT $10^2$
	Absence of <i>E. coli</i> in 10 g

### Absence of Objectionable Microorganisms

See *Introduction* under [Microbiological Procedures for Absence of Specified Microorganisms—Nutritional and Dietary Supplements](#) { 2022 }. Absence of one or more species of objectionable microorganisms is required in some individual monographs.

**Test for Aflatoxins**— Dietary and nutritional articles containing botanical products with a history of mycotoxin contamination are also typically tested for aflatoxins, especially if the material is obtained from roots or rhizomes. See [Articles of Botanical Origin](#) { 561 } for the details of a test for aflatoxins. Where necessary, this test is included in the individual monograph.

**Solid Oral Dosage Forms**— Among all dosage forms, solid oral dosage forms present the lowest microbiological risk because of their method of manufacture, low water activity, and route of administration. When justified, reduced microbiological testing may be appropriate.

**Other Concerns**— The presence of some microorganisms in articles can be an indicator of processes that are not under microbiological control. For example, [Purified Water](#) used at some stage of the manufacture of these products might contain a typical flora of Gram-negative microorganisms. As with pharmaceutical products, inadequate processing of water and poor maintenance of water systems may result in the contamination of processed formulations by Gram-negative microorganisms.

**Auxiliary Information**— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	<a href="#">Radhakrishna S Tirumalai, Ph.D.</a> Principal Scientific Liaison 1-301-816-8339	(GCM2010) General Chapters - Microbiology

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