

Evaluation, Validation and Implementation of New Microbiological Testing Methods

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OF NEW MICROBIOLOGICAL TESTING METHODS

Table of Contents

Part One: Selection of New Microbiological Methods

1.0 Introduction	1
1.1 Scope of Document	1
1.2 Purpose of Document	1
1.3 Overview of Document Structure	2
2.0 Technology Overview	2
2.1 Generic Description of Types of Microbiological Methods	2
2.2 Technology Review	3
2.3 Growth-based Technologies	3
2.3.1 ATP Bioluminescence	3
2.3.2 Colorimetric Detection of CO ₂ Production	4
2.3.3 Measurement of Change in Head Space Pressure	4
2.3.4 Impedance	4
2.3.5 Biochemical Assays	4
2.4 Viability-based Technologies	6
2.4.1 Solid Phase Cytometry	6
2.4.2 Flow Fluorescent Cytometry	6
2.5 Artifact-based Technologies	7
2.5.1 Fatty Acid Profiles	7
2.5.2 Mass Spectrometry	7
2.5.3 ELISA	7
2.5.4 Fluorescent Probe Detection	7
2.5.5 Bacterial Endotoxin-Limulus Amebocyte Lysate Test	7
2.6 Nucleic Acid-based Technologies	8
2.6.1 DNA Probe	8
2.6.2 Ribotyping/Molecular Typing	8
2.6.3 Polymerase Chain Reaction	9
3.0 Regulatory Review	10
3.1 General Classification of Microbiological Testing	10
3.1.1 In-Process Tests	10
3.1.2 Product Tests	10
3.1.3 Qualitative Tests	10
3.1.4 Quantitative Tests	10
3.2 Compendial Microbiological Test Method References	10
3.2.1 Water for Pharmaceutical Purposes	10
3.2.2 Antimicrobial Effectiveness Testing	10
3.2.3 Microbial Limit Testing	11
3.2.4 Sterility Testing	11
3.2.5 Environmental Monitoring	11
3.2.6 Microbial Identification	11

3.3 Changing a Microbiological Test Method	11
3.3.1 The Regulatory Perspective on the Introduction of New Microbiological Test Methods	11
3.3.2 The Compendial Perspective	12
3.4 Obtaining Compendial Change	13
3.5 Regulators Assessment of New Microbiological Methods	13

Part Two: How to Validate and Implement a New Microbiological Test Method

4.0 The Validation Process	15
4.1 The Equipment Qualification Model	16
4.1.1 Vendor/Specification Requirements	16
4.1.1.1 Test Method Selection	17
4.1.1.2 Vendor Selection	17
4.1.2 Validation Plan Design	18
4.1.3 Installation Qualification	18
4.1.4 Operation Qualification	20
4.1.5 Performance Qualification	20
4.2 Validation Criteria	21
4.2.1 Preparation of Test Samples	23
4.2.2 Variability of Microbiological Methods: Special Note	25
4.2.2.1 Sample Distribution Error	25
4.2.2.2 Cell Morphology	25
4.2.2.3 Metabolic Activity	25
4.2.3 Protocol Design Using Recommended Validation Criteria	26
4.3 Special Considerations for the Validation of Microbiological Methods	29
4.3.1 Use of Multiple Pieces of the Same Equipment within the Laboratory and Company	29
4.3.2 Unique Testing Requirements for Microbiological Equipment	30
5.0 Glossary of Terms	32
6.0 References	39

PART ONE:

Selection of New Microbiological Methods

1.0 INTRODUCTION

1.1 Scope of Document

This document is intended to provide guidance for the successful evaluation, validation, and implementation of new microbiological methods needed by the pharmaceutical, biotechnology, and medical device industries to assure product quality. Applications for these methods include but are not limited to Microbial Limit Testing, Sterility Testing, Anti-microbial Effectiveness Testing, Microbiological Monitoring of Clean Rooms and Other Controlled Environments, Water for Pharmaceutical Purposes Monitoring, and Microbial Characterization and Identification.

The intended audience of this report is microbiologists who are responsible for the validation of the microbiological test methods used in the routine microbiology testing laboratory. The document should be of interest to suppliers of testing equipment, microbiology managers and supervisors, validation specialists, quality control personnel responsible for the approval of validation protocols and the release of product, and regulatory agencies.

1.2 Purpose of Document

Microbiological testing plays an ever increasing role in the pharmaceutical laboratory. In response to this, a variety of new methodologies have emerged in recent years which automate existing methods, make use of surrogate markers for growth, or are based on wholly new technologies. These new methodologies offer significant improvements in terms of the speed, accuracy, precision, and specificity with which testing can be performed.

The majority of testing performed today relies on century-old methods, based on the recovery and growth of microorganisms, using solid or liquid microbiological growth media. This is true in part because these methods can be very effective and have a long history of application in both industrial and clinical settings. However, they are often limited by

slow microbial growth rates, the unintended selectivity of microbiological culture, and the inherent variability of microorganisms in their response to culture methods. In spite of the limitations of current culture methods, acceptance of new and potentially superior methods is often slow. We believe this is due in part to a lack of clear guidance regarding the demonstration of their equivalence to existing methods acceptable to regulatory agencies and validation of the equipment associated with the new methods. This technical report hopes to provide guidance to assist with the evaluation, validation, and implementation of the newer microbiological methods.

Considerable guidance can be found regarding the validation of chemical methods. Examples include USP General Informational Chapter <1225> *Validation of Compendial Methods* (1), and a recent publication by the International Conference on Harmonization (ICH) *Validation of Analytical Methods* (2). These publications provide very specific instruction regarding the demonstration of new analytical chemistry methods and their equivalence to existing methods. In contrast, virtually no guidance specific to microbiological testing has been published. Possible exceptions are the ASM Cumitech publication *Verification and Validation of Procedures in the Clinical Microbiology Laboratory* (3), that addresses pathogen isolation and identification and antimicrobial susceptibility testing, and the new USP General Information Chapter <1227> *Validation of Microbial Recovery from Pharmacopeial Articles* (4). However, more guidance is needed. Because microbiological methods are inherently different than chemical assays, this lack of agreed upon demonstration criteria can present serious obstacles to their implementation.

When instrumentation is developed for existing microbiological methods to automate sample handling, result reading, or data management or to miniaturize the test procedure, it is not difficult to demonstrate the equivalency of the alternate method using guidelines developed for chemical assays, because the test remains essentially the same. In a similar fashion, when a new technology continues to rely on the measurement of microbial growth (e.g., impedance, ATP bioluminescence or other metabolic changes in a

microbial culture), equivalence can be readily demonstrated. However, when a new method is based on novel technology without direct ties to the existing method (e.g., microbial identification by DNA amplification versus patterns of biochemical reactions, or counting fluorescent- labeled bacterial cells instead of colony-forming units on an agar plate), demonstration of equivalency may require a new application of the validation principles, although the method provides higher quality results.

The application of these new technologies may be compared to the replacement of the Most Probable Number-multiple fermentation tubes with membrane filtration methods for counting coliforms in water, and the change from solely morphological features to physiological and biochemical characteristics to DNA-based methods for the identification of bacteria. For example, the ability to count individual, viable, fluorescent-labeled microbial cells by scanning a membrane with a laser should be superior to counting colony-forming units; the nucleic acid contained in a microorganism is conservatively unique to that species, i.e., genotypic identification, hence is superior to patterns of biochemical reactions that are currently used to identify bacteria, i.e., phenotypic identification.

This document, was developed as a collaborative effort amongst representatives from test manufacturers, the pharmaceutical and device industry, standards organizations, and regulatory agencies. It is intended to provide a general approach to the introduction of new microbiology methods in a government-regulated environment. It is anticipated that by providing agreed upon performance standards, the development, demonstration and implementation of superior methods will be greatly accelerated.

1.3 Overview of Document Structure

This document was written to establish industry-wide criteria on what constitutes an acceptable microbiology test and how to prove it to the satisfaction of a regulatory agency.

The document is divided into two major sections. In the Method Selection section, a review of microbiology testing methods is followed by an overview of compendial applications which make use of these tests. A discussion of requirements for regulatory acceptance and economic justification completes the section. In the Validation section, criteria used for validation and demonstration of equivalence are defined, and approaches to validation methods and documentation are described. Finally, a decision tree, glossary of commonly used terms, and a bibliography are provided to present an overview of the entire process.

2.0 TECHNOLOGY OVERVIEW

2.1 Generic Description of Types of Microbiological Methods

Microbiological test methods can be divided into three general categories, based on their function: 1) detection of the presence or absence of microorganisms in a test sample, 2) enumeration of microorganisms present in a test sample, and 3) characterization and identification of microorganisms either present in test samples or from a pure culture.

Presence/absence tests may be designed to detect diverse types of microorganisms, as in sterility test methods, or may be intended for detection of specific microbial species or genera, as in the tests for Pharmacopeial Indicator Organisms. USP, Ph. Eur. and JP have the same set of indicator organisms, i.e., *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella* species.

The results obtained when testing samples by different enumeration methods based on classical microbiological techniques may vary greatly, depending on the test conditions, e.g., media type and incubation temperature, conditions, and duration.

An example is the difference seen in plate counts from water samples using a low nutrient medium

such as R2A agar with a 5 to 7 day incubation period at ambient temperature, versus Soybean – Casein Digest agar incubated for 48 hours at 30 to 35°C. Significant differences in counts may also be seen when comparing non-growth based direct detection methods with classical methods, with higher counts obtained by the former due to suboptimal culture conditions in microbiological media.

Many types of microbial characterization and identification test methods are used, ranging from classical Gram's staining and simple manual tests for specific enzymes associated with bacterial cells such as catalase and cytochrome oxidase, to compendial methods using the appearance of microbial colonies on selective/differential growth media, to semi-automated biochemical profiling systems, to methods based on the detection of specific nucleic acid sequences or cell membrane fatty acid composition.

The test methods vary greatly in their cost, ease of use, duration, and performance as measured by analytical parameters such as specificity, sensitivity, reproducibility, and ruggedness.

2.2 Technology Review

As discussed earlier, the century-old methods developed by the pioneering microbiologists Pasteur, Koch, Lister, and many others is the technology base for current compendial microbiological test methods. These methods are based on providing conditions to allow microbial cells present in test samples to grow and replicate sufficiently to allow their detection, typically by visual examination of a plate or broth. Specific compendial tests will be discussed later in the section on Regulatory Review, so the focus of this section will be on newer technologies.

New technology for microbiology quality assurance testing is a rapidly developing area. The Task Force has attempted to include all technologies with current application in the pharmaceutical industry. However, exclusion of a technology or

application of a technology is not intentional, and does not imply that the technology is not suitable. This review is simply the best available information at the time. Without doubt, further developments will occur during the lifetime of this document, which will mean that new technologies or new applications of existing technologies will be brought to market. For convenience, the technologies are divided into 1) growth-based technologies, 2) viability-based technologies, 3) cellular component or artifact-based technologies, and 4) nucleic acid-based technologies.

Reference to representative suppliers (see Tables 1, 2, 3, and 4) is merely given as examples compiled by the Task Force members and does not infer commercial endorsement on the part of the Task Force or PDA.

2.3 Growth-based Technologies

These methods are based on the measurement of biochemical or physiological parameters that reflect the growth of the microorganisms.

2.3.1 ATP Bioluminescence

The presence of Adenosine Triphosphate (ATP) is a well-documented marker for cell viability. All cells store energy in the form of ATP. An increase in cell numbers results in an increase in ATP levels. ATP bioluminescence utilizes the luciferin-luciferase reaction of the firefly to detect the presence of ATP by measuring the light emitted. The presence or absence of microbial contamination in a sample can be determined by measuring the increase in ATP levels following incubation. ATP bioluminescence reduces the test time to approximately one third of that taken by the traditional method. This is because it is a more sensitive endpoint detection system, using sensitive chemistry and instrumentation, rather than relying on the human eye. This means that identical principles are in use, i.e., multiplication of microorganisms, but the amount of replication required for detection is significantly reduced: 10^3 cells per mL as opposed to 10^7 or greater, to determine turbidity or colonies on a plate.

2.3.2 Colorimetric Detection of CO₂ Production

Test samples are placed in culture bottles for monitoring. The samples are incubated, agitated, and monitored for the presence of microorganisms. These systems use colorimetric detection of CO₂ production from the growth of organisms. Some systems will detect color change, flag a positive sample, and alert the user. These systems are often referred to as non-invasive microbial detection systems and can accommodate a large number of samples. Although commonly used clinically for blood cultures, the method could be used for sterility testing.

2.3.3 Measurement of Change in Head Space Pressure

These systems are based on non-invasive, continuous, automated monitoring of microbial cultures. Electronic transducers are used to sense positive or negative pressure changes in the head space of each culture bottle. These changes are caused by microbial growth. If significant production and/or consumption of gas is detected, samples are flagged as positive. Large quantities of samples can be placed into these instruments for testing with frequent monitoring of the head space pressure. Although commonly used clinically for blood cultures, the method could also be used for sterility testing.

2.3.4 Impedance

Impedance measures microbial activity by electrical methods. These instruments measure ionic changes occurring within the growth media as bacteria multiply. The impedance detection time is inversely proportional to the number of microorganisms present at initial inoculation. Bacteria metabolize larger, weakly charged molecules and produce smaller highly charged by-products. For example, large weakly charged molecules such as proteins are hydrolyzed to many highly charged amino acids. Two electrodes are then used to measure these ionic changes in the broth or agar culture.

2.3.5 Biochemical Assays

Microbial cell suspensions of pure cultures are tested with a series of biochemical substrates. Microorganisms are known to have particular reactions to these biochemicals, e.g., carbohydrate utilization. By matching the biochemical results with a database of corresponding results, one can determine the identification of the organism being tested. Many of these methods are performed and recorded manually. High volume, automated instruments are also commercially available to read miniature cultures and identify the microorganisms from the pattern of reactions in the database.

In the following tables, presence/absence, enumeration and identification methods are designed as P, E, and I respectively.

Table 1: Growth-based Microbiological Methods

Technology	Representative Commercial Products	Principal Applications
ATP Bioluminescence	SteriScreen and MicroStar Rapid Microbiology and MicroCount Digital Systems (Millipore Corp, Bedford, MA & RapiScreen (Celsis, Evanston, IL)	Raw material & product screening, water monitoring, pre-sterile filtration bioburden monitoring (E) Sterility testing (P)
Colorimetric Detection of Carbon Dioxide Production Headspace Pressure	Bact/Alert (Organon-Teknika, Durham, NC) & ESP Microbial Detection System (AccuMed International, Detroit, MI)	Sterility testing (P)
Biochemical & Physiological Reactions	API Systems (bioMerieux, Hazelwood, MO), BIOLOG Systems (Biolog, San Diego, CA) & VITEK System (bioMerieux, Hazelwood, MO)	Microbial identification (I)
Impedance /Conductivity	Bactometer (bioMerieux, Hazelwood, MO) & Malthus Microbial Detection System (Malthus Diagnostics North Ridgeville, OH)	Bioburden monitoring, antimicrobial effectiveness testing (E)
Spiral plating	Spiral Plating System (Spiral Biotech, Bethesda, MD)	Bioburden monitoring (E)
Hydrophobic Grid Membrane Filter Methods	Iso-Grid (QA LifeScience, San Diego, CA)	Bioburden monitoring (E) Pathogen monitoring (E)
Pour Plate Method	Conventional	Bioburden monitoring, water monitoring, antimicrobial effectiveness testing (E)
Most Probable Number Multiple - Tube Method	Conventional	Bioburden monitoring, water monitoring, D-Value analysis (E)
Membrane Filtration Method	Conventional	Bioburden monitoring (E) Sterility testing (P), Water monitoring (E)

2.4 Viability-based Technologies

2.4.1 Solid Phase Cytometry

The method of solid phase cytometry utilizes membrane filtration to separate potential microbial contaminants from filterable samples prior to labeling of the captured cells with a universal viability substrate. Once within the cytoplasm of metabolically active microorganisms, the non-fluorescent substrate is enzymatically cleaved to release free fluorochrome by the hydrolytic enzyme esterase. Only viable microorganisms with membrane integrity have the ability to retain the marker used in the assay. The filter is then automatically scanned by a laser based detector, and the number of fluorescent-labeled cells immediately reported.

Since the method eliminates the need for cell multiplication, sensitivities to the single cell level independent of volume filtered, are possible for all microbial cells including spores, as well as stressed cells and fastidious organisms. Since a much more sensitive end point detection mechanism is employed when compared to growth based procedures, near real-time results are available for a full range of viable microorganisms.

2.4.2 Flow Fluorescence Cytometry

In addition to filterable products, this approach has now been applied to non-filterable products by employing similar labeling chemistries to highly sensitive flow cytometers.

Table 2: Viability-based Microbiological Methods

Technology	Representative Commercial Products	Principal Applications
Direct Epifluorescent Filter Microscopy	Direct Epifluorescent Filter Technique (DEFT) Automate Counting System (Micro-Measurements, Saffron Walden, Essex, United Kingdom)	Bioburden monitoring (E)
Membrane Laser Scanning Fluorescence Cytometry	Scan RDI (Chemunex, Monmouth Junction, NJ)	Water monitoring, Bioburden monitoring, Environmental monitoring (E) Sterility testing (P)
Fluorescence Flow Cytometry	DCount (Chemunex, Monmouth Junction, NJ)	Bioburden monitoring (E)

2.5 Artifact-based Technologies

2.5.1 Fatty Acid Profiles

Fatty acid analysis by high resolution gas chromatographs determines the fatty acid composition of unknown isolates. Databases are then searched for a match with known isolates. Cellular fatty acid compositions are stable and conserved. This technology is able to be utilized because of the large number of fatty acids which occur in bacteria and the reproducibility of fatty acid composition within a taxonomic group. Samples are saponified, methylated, and extracted manually before being placed onto the gas chromatogram and the fatty acid methyl esters (FAMES).

2.5.2 Mass Spectrometry

Matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry may be used to provide rapid identification and characterization of intact microbial cells. Different microorganisms generate different charged molecular weight patterns or spectra that can be used to identify organisms to genus, species, and in some cases strains.

2.5.3 ELISA

Enzyme linked immunosorbent assay (ELISA) is a labeling technique for demonstrating the presence, or absence, of an antibody or antigen. The method depends on the use of immunoreagents coupled to an enzyme. A separation step of free and bound conjugate is then necessary to obtain a result. There are several components involved to carry out an ELISA: solid phase, which is necessary to allow for separation of the bound and free conjugate; enzyme, which is used in the conjugate where it is labeled to an antigen or antibody; and substrate, used to detect end product.

2.5.4 Fluorescent Probe Detection

Fluorescent probes are designed to bind to specific target sites on or in cells, e.g., antibodies or nucleic acid and contain a molecule capable of fluorescing when stimulated by an energy source such as a coherent light (laser).

2.5.5 Bacterial Endotoxin-*Limulus Amebocyte* Lysate Test

The lysate of the blood cells of the American horseshoe crab, *Limulus polyphemus* has a very sensitive clotting system triggered by the lipopolysaccharide of the cell wall of gram-negative bacteria. This test replaced the rabbit pyrogen test and is an example of where an alternate test became the compendial test.

Table 3: Artifact-based Microbiological Methods

Technology	Representative Commercial Products	Principal Applications
Fatty Acid Profiles	Sherlock Microbial Identification System (MIDI, Newark, DE)	Microbial identification (I)
MALDI-TOF Mass Spectrometry	Kratos Analytical Systems (Manchester, UK) Perseptive Biosystems Voyager – DE (Framingham, MA)	Microbial identification (I)
Fluorescence Antibody Techniques	RBD-2000 (Advanced Analytical Technologies, Ames, IA)	Pathogen monitoring (P, E & I)
Enzyme-linked Immunosorbent Assay	VIDAS & Mini-VIDAS ELISA Systems (bioMerieux, Hazelwood, MO), Tecra - Salmonella ELISA (International BioProducts, Redmond, WA), Salmonella-Tek ELISA (Organon-Teknika, Durham, NC)	Pathogen monitoring (I)
Latex Agglutination	Bactigen Salmonella-Shigella Test (Wampole Laboratories, Cranbury, NJ)	Pathogen monitoring (P & E)
Limulus Amebocyte Lysate-Endotoxin Assay	Pyrogent Gel Clot (BioWhittaker, Walkerville, MN) & Pyrotell (Associates of Cape Cod, Falmouth, MA)	Pyrogen monitoring (P & E)
Gram's Stain	Conventional	Differentiates bacteria by Gram reaction (I)

2.6 Nucleic Acid-based Technologies

2.6.1 DNA Probe

DNA hybridization assays specifically designed for the detection of target organisms utilize solid support devices for hybridization chemistry and colorimetric detection via an enzyme. Steps involved are sample lysis, hybridization, hybrid capture, enzyme label, color development, and detection.

2.6.2 Ribotyping/Molecular Typing

This technique characterizes and definitively identifies organisms using restriction fragments of nucleic acids from bacterial genomes. The size-separated DNA restriction fragments are hybridized to a ribosomal RNA probe. A chemiluminescent substrate is applied. A camera converts the luminescing DNA fragments to digital information. This digital information is captured and data

are extracted. A pattern is then generated. This pattern is compared to a database of patterns from known bacterial isolates. The ribotype pattern is a stable epidemiological marker that provides definitive taxonomic information as well as species discrimination.

2.6.3 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) targets a specific fragment of DNA which is highly conserved and therefore stable. This provides a highly reliable indicator of the organism's presence. PCR quickly (2-3 hours) provides millions of copies of this specific fragment by combining sample DNA with polymerase, nucleotides, and primers that are specific for a given nucleotide sequence.

The mixture is placed into a thermocycler and is heated and cooled through many cycles. Heating denatures the DNA into single strands. As cooling takes place, the primers anneal to the target DNA sequence. The polymerase enzyme then uses the nucleotides to extend the primers. These steps create two copies of the target fragment during one cycle. Repeated cycles of denaturing, annealing, and extending allow for exponential production of target DNA fragments. If the target sequence is not present in the sample, detectable amplification does not occur. Electrophoresis of the PCR product is usually used to detect for the presence of the specific fragment. PCR technology allows for far more rapid, sensitive, and accurate detection than conventional methods.

Table 4: Nucleic Acid-based Microbiological Methods

Technology	Representative Commercial Products	Principal Applications
Nucleic Acid Probe	Gene-Trak System (Gene-Trak Systems, Hopkinton, MA) & Gene-Probe System, (Gene-Probe, San Diego, CA)	Pathogen monitoring (P)
Polymerase Chain Reaction - DNA Amplification	BAX ® Microbial Identification System (Qualicon, Wilmington, DE) Probelia System (BioControl Systems, Bellevue, WA)	Pathogen monitoring (P)
16S rRNA Sequencing Techniques	MicroSeq. 16S rDNA Bacterial Identification System (PE Applied Biosystems, Foster City, CA)	Microbial identification (I)
Automated Ribotyping	RiboPrinter ® System (Qualicon, Wilmington, DE)	DNA Fingerprinting for microbial characterization & identification (I)

3.0 REGULATORY REVIEW

3.1 General Classification of Microbiological Testing

There is a range of microbial tests conducted in the microbiological testing laboratory, and their regulatory requirements differ, based on the criticality of the test to the final pharmaceutical drug product and the intended route of administration of the product. The range of tests can be classified as follows:

3.1.1 In-process Tests

Most in-process testing is to assure the adequacy of components, environments, equipment and processing steps to control microbial contamination. These tests are often required to demonstrate conformance to current Good Manufacturing Practices (cGMPs). Changes to microbiological tests based on new technology will be evaluated scientifically. However, this evaluation is less likely to be done unless the change is filed in the product application. Many microbiological test methods used in in-process monitoring are not included in the original product application files, and changes in these methods would not be filed when the new methods are introduced. It is recommended that changes to new microbiology technologies be filed as changes in the product application. It should be noted that most in-process microbiological tests are quantitative.

When evaluating test data, comparisons are valid only when the methods remain consistent. This should not, however, prevent new technology from being considered as a replacement for an older method. During test method validation, a period of comparison may permit the transition from old to new microbiological methods. New acceptance criteria may need to be established, e.g., a viability-based enumeration may give higher recoveries than a membrane filtration method.

3.1.2 Product Tests

Microbiological tests are conducted for product release and also as part of the stability protocol.

These tests may provide qualitative or quantitative results that may be used for different purposes, e.g., to demonstrate that the product the absence of an objectionable microorganism or meets a Microbial Limit.

3.1.3 Qualitative Tests

Product release microbiology tests are often qualitative and assess a limit for conformance to established acceptance criteria. Comparisons of new and old technology for qualitative tests are based on the ability of the test to detect the qualitative attribute. For example, sterility tests evaluate a qualitative attribute and may sacrifice quantitative results in order to enhance sensitivity of the test.

3.2 Compendial Microbiological Test Method References

3.2.1 Water for Pharmaceutical Purposes

Types of water, methods and specifications for testing them are listed in U.S. Pharmacopeial Informational Chapter <1231> *Water for Pharmaceutical Purposes* (5). The United States Pharmacopeia (USP) references Standard Methods for the Examination of Water and Waste Water, (APHA) 20th edition, 1998 (6) for information on specific test methods. In supplement 2000 of the Pharmacopeia Europa (Ph. Eur.) there will be two monographs on water. One is water for injections, subdivided into sections on water for injection in bulk and a section for sterilized water for injections. The second monograph is on purified water. This monograph is also divided into two sections: purified water in bulk and purified water in containers. For the microbiological examination (if relevant) soybean-casein digest agar and not R2A or Plate Count agar is prescribed.

3.2.2 Antimicrobial Effectiveness Testing

Antimicrobial preservatives are substances added to multi-use products to protect them from mi-