

⟨1227⟩ VALIDATION OF MICROBIAL RECOVERY FROM PHARMACOPEIAL ARTICLES

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INTRODUCTION

▲This chapter provides guidelines for the validation of recovery methods for the estimation of the number of viable microorganisms, the detection of indicators or specified microorganisms, and the sterility testing of pharmacopeial articles. The test procedures in *Antimicrobial Effectiveness Testing* ⟨51⟩, *Sterility Tests* ⟨71⟩, *Microbial Enumeration Tests* ⟨61⟩, and *Tests for Specified Microorganisms* ⟨62⟩ are considered validated. However, use of compendial methods requires establishment of suitability of the method demonstrating recovery of the challenge organisms in the presence of the product. Alternatives/modifications to these recovery procedures beyond what are described in these chapters (such as dilution, chemical or enzymatic neutralization, and membrane filtration) require validation.▲ (USP 1-Dec-2019) It is generally understood that if a product possesses antimicrobial properties because of the presence of a specific preservative or because of its formulation, this antimicrobial property must be neutralized to recover viable microorganisms. This neutralization may be achieved by the use of a specific neutralizer, by dilution, by a combination of ▲▲ (USP 1-Dec-2019) dilution, ▲filtration, and rinsing,▲ (USP 1-Dec-2019) or by any combination of these methods. ▲When the product displays intrinsic antimicrobial activity for a given microorganism and, given this antimicrobial activity, the risk of microbial contamination is low, the method could be considered as fit for the purpose of providing a strong rationale.▲ (USP 1-Dec-2019)

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INFLUENTIAL FACTORS

Several factors affect the measurement of a test solution's antimicrobial activity, and these must be considered in the validation design. They include the nature of the microorganisms used as challenge organisms, preparation of the inoculum of challenge organisms, specific conditions of the test, and conditions of recovery. These factors also affect the validation of recovery methods for aqueous or nonaqueous products, irrespective of their antimicrobial properties; thus, all test methods should be validated with these factors in mind.

The nature of the challenge microorganism exerts a strong effect upon the response to the antimicrobial agent, and so upon the neutralization required for recovery. Represented among these organisms in compendial tests are gram-positive bacteria, gram-negative bacteria, ▲anaerobic bacteria,▲ (USP 1-Dec-2019) yeasts, and molds. Each organism to be used in the test must be included in the validation.

The preparation of the inoculum of challenge microorganisms also affects the testing of products having antimicrobial properties. The growth and preparation of the challenge organism determines the physiological state of the cell. This state has a direct influence on the results of any test of antimicrobial efficacy. Microbial tests do not use individual cells; rather, populations of cells are harvested for study. The data generated from these studies are less variable if the cell populations are homogeneous. Liquid cultures or confluent growths on solid medium are best suited for reproducible culture preparation. The conditions of organism preparation and storage must be standardized for the neutralizer evaluation and should reflect the conditions of the antimicrobial assay.

The specific conditions of the test, including buffers used, water, light conditions, and temperature, must be reproduced in the validation study. All test conditions also should be standardized and performed in the validation study exactly as performed in the test.

The conditions of microbial recovery are among the most crucial in accurately estimating the number of microorganisms present in a test solution. The first consideration is the recovery medium used to support the growth of survivors. This concern is discussed in detail below. The second consideration is the incubation conditions. Optimal conditions for growth must be present to ensure complete growth and reproducible results.

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METHODS OF NEUTRALIZING ANTIMICROBIAL PROPERTIES

Three common methods are used to neutralize antimicrobial properties of a product: 1) chemical inhibition, 2) dilution, and 3) filtration and ▲rinsing.▲ (USP 1-Dec-2019)

Chemical ▲Neutralization▲ (USP 1-Dec-2019)

Table 1 shows known neutralizers for a variety of chemical antimicrobial agents and the reported toxicity of some chemical neutralizers to specific microorganisms. However, despite potential toxicity, the convenience and quick action of chemical inhibitors encourage their use. Chemical ▲neutralization of antimicrobial agents▲ (USP 1-Dec-2019) is the preferred method for the antimicrobial efficacy test. The potential of chemical ▲neutralizers▲ (USP 1-Dec-2019) should be considered in the membrane filtration and the direct ▲inoculation▲ (USP 1-Dec-2019) sterility tests. Antibiotics may not be susceptible to neutralization by chemical means, but rather by enzymatic treatment (e.g., penicillinase). These enzymes may be used where required.

Table 1. Some Common Neutralizers for Chemical Antimicrobial Agents (USP 1-Dec-2019)

Neutralizer	Antimicrobial Class (USP 1-Dec-2019)	Potential Action of Antimicrobial Agents (USP 1-Dec-2019)
Bisulfate	Glutaraldehyde, mercurials	Non-sporing bacteria
Dilution	Phenolics, alcohol, aldehydes, sorbate	—
Glycine	Aldehydes	Growing cells
Lecithin	Quaternary ammonium compounds (QACs), parabens, bis-biguanides	Bacteria
Mg ⁺² or Ca ⁺² ions	EDTA	—
Polysorbate	QACs, iodine, parabens	—
Thioglycollate	Mercurials	Staphylococci and spores
Thiosulfate	Mercurials, halogens, aldehydes	Staphylococci

Dilution

A second approach to neutralizing antimicrobial properties of a product is by dilution, because the concentration of a chemical antimicrobial agent (USP 1-Dec-2019) exerts a large effect on its potency. The relationship between concentration and antimicrobial effect differs among bactericidal agents but is constant for a particular antimicrobial agent. This relationship is exponential in nature, with the general formula:

$$C^{\eta}t = k$$

- C = concentration of the antimicrobial agent (USP 1-Dec-2019)
 η = concentration exponent (dilution coefficient), (USP 1-Dec-2019) the slope of the plot of $\log t$ versus $\log C$
 t = time required to kill a standard inoculum
 k = a constant

Antimicrobial agents with high η values are rapidly neutralized by dilution, whereas those with low η values are not good candidates for neutralization by dilution (see Table 2).

Table 2. Concentration Exponents for Some Common Antimicrobial Agents

Representative Antimicrobial Agent	η Values	Increased Time Factor (x) to Kill Microorganisms When the Concentration Is Reduced to:	
		One-Half	One-Third
Phenolics	6	64	729
Alcohol	10	1024	59,000
Parabens	2.5	6	16
Chlorhexidine	2	4	8
Mercury compounds	1	2	3
Quaternary ammonium compounds	1	2	3
Formaldehyde	1	2	3 (USP 1-Dec-2019)

Membrane Filtration

An approach that is often used, especially in sterility testing, is neutralization by membrane filtration. This approach relies upon the physical retention of the microorganism on the membrane filter, with the antimicrobial agent passing through the filter into the filtrate. The filter is then incubated for recovery of viable microorganisms. However, filtration alone may not remove sufficient quantities of the antimicrobial agent (USP 1-Dec-2019) to allow growth of surviving microorganisms. Adherence of residual antimicrobial agents to the filter membrane may cause growth inhibition. Filtration through a low-binding filter material, such as polyvinylidene difluoride, helps to minimize this growth inhibition. Additionally, the preservative may be diluted or flushed from the filter by rinsing with a non-toxic fluid, such as diluting Fluid A (see *Sterility Tests* <71>, *Diluting and Rinsing Fluids for Membrane Filtration* for diluting fluid compositions). Chemical neutralizers in the rinsing fluid can ensure that any antimicrobial residue on the membrane does not interfere with the recovery of viable microorganisms.

Change to read:**VALIDATION OF NEUTRALIZATION METHODS—RECOVERY COMPARISONS**

A validated method for neutralizing the antimicrobial properties of a product must meet two criteria: neutralizer efficacy and neutralizer [▲]non-toxicity. [▲](USP 1-Dec-2019) The validation study documents that the neutralization method employed is effective in inhibiting the antimicrobial properties of the product (neutralizer efficacy) without impairing the recovery of viable microorganisms ([▲]lack of [▲](USP 1-Dec-2019) neutralizer toxicity). Validation protocols may meet these two criteria by comparing recovery results for treatment groups.

The first is the test group, in which the product is subjected to the neutralization method, then a low level of challenge microorganism [less than 100 colony-forming units (cfu)] is inoculated for recovery. The second is the peptone control group, in which the neutralization method is used with peptone, or diluting *Fluid A* (see *Sterility Tests* <71>, *Diluting and Rinsing Fluids for Membrane Filtration*), as the test solution. The third is the viability group, in which the actual inoculum is used without exposure to the neutralization scheme. Similar recovery between the test group and the peptone group demonstrates adequate neutralizer efficacy; similar recovery between the peptone group and the viability group demonstrates adequate neutralizer [▲]non-toxicity. [▲](USP 1-Dec-2019)

In principle, the protocol must show that recovery of a low inoculum (less than 100 cfu) is not inhibited by the test sample and the neutralization method. Validation protocols may meet these two criteria by comparing recovery among three distinct test groups: 1) neutralized product with inoculum, 2) challenge inoculum control in buffered solution, and 3) inoculum in the absence of product or neutralizer. This can be established by directly comparing the result in the treated solution (1) to the inoculum (3) above. If the growth on the treated solution is not comparable to the growth on the inoculum group, it should be determined whether the neutralization method itself is toxic to the microorganisms.

Recovery on Agar Medium

In the tests under <51> and <61>, [▲](USP 1-Dec-2019) the number of viable challenge microorganisms in the product is estimated [▲](USP 1-Dec-2019) by calculating the concentration of cfu per milliliter by the plate count method. A design for validating neutralization would incorporate the treatment groups as described under *Validation of Neutralization Methods—Recovery Comparisons*. At least three independent replicates of the experiment should be performed, and each should demonstrate [▲]a mean count of any of the test organisms not differing by a factor greater than 2, i.e., 50%–200% recovery, from the value of the control in the absence of product. If it is necessary to solubilize the test [▲]sample, [▲](ERR 1-Dec-2019) the effects of the solubilization method on viable microorganisms must be determined. This situation can occur when testing ointments, suspensions, or other articles. [▲](USP 1-Dec-2019)

If a greater number of replicates is required in the validation study, the comparisons may be evaluated by transforming the numbers of cfu to their logarithmic values and analyzing the data statistically by the Student *t* test (pairwise comparisons) or by analysis of variance (ANOVA) (for comparing all groups). If ANOVA is used, and significant differences among the populations are determined, a test such as Dunnett's test may be used, with the peptone group used as the control group.

Recovery by Membrane Filtration

This validation follows the procedure described in [▲]*Sterility Tests* <71>, *Method Suitability Test*, [▲](USP 1-Dec-2019) with the exception of plating on solid medium to quantitate recovery. [▲]It should be emphasized that quantitative recovery is not required to demonstrate sterility test suitability. It only requires a qualitative assessment (visual turbidity). [▲](USP 1-Dec-2019) Three 100-mL rinses are assumed, but the volume and number of rinses are subject to validation. [▲]A maximum of five 100-mL washes should be used for routine testing even if during method suitability it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity. [▲](USP 1-Dec-2019) Each validation run should be performed independently at least three times.

In the test solution group, the product is passed through the membrane filter, followed by two 100-mL portions of diluting-neutralizing fluid. After the second rinse has been filtered, a final 100-mL portion containing less than 100 cfu of the specific challenge microorganism is passed through the filter. This filter is then placed on the appropriate agar recovery medium and incubated for recovery.

The inoculum is directly plated onto the solid medium. It is possible that filtration will lead to reduced recovery of the challenge microorganism, either through inherent toxicity of the membrane or by adherence of the microorganism to the filtration vessel walls. A control group can be used to evaluate this component of membrane filtration validation. Diluting *Fluid A* is used as the dilution medium without exposing the filter to the product. After addition of the low-level inoculum to the final rinse, the filter is plated as above. Technique-specific loss of microorganisms can be estimated by comparing the recovery in the diluting *Fluid A* (see *Sterility Tests* <71>, *Diluting and Rinsing Fluids for Membrane Filtration*) group to the inoculum count.

It is assumed in this discussion that the test sample can be filtered. If it is necessary to solubilize the test sample, the effects of the solubilization method on viable microorganisms must be determined. This situation can occur when testing ointments, suspensions, or other articles.

The method can be considered validated if the recovery rate in the three independent replicates is similar for the test solution and the diluting *Fluid A* (see *Sterility Tests* <71>, *Diluting and Rinsing Fluids for Membrane Filtration*) control.

Recovery in Liquid Medium

It is assumed in *Sterility Tests* <71>, *Test for Sterility of the Product to be Examined, Direct Inoculation of the Culture Medium* that the recovery medium will allow for growth of all surviving microorganisms. The broth in that test must serve both to neutralize any antimicrobial properties of the test solution and to support the growth of the microorganisms. The treatment groups

described under *Validation of Neutralization Methods—Recovery Comparisons* can be used for validation of the recovery method, with the proportions of product and recovery medium varied to achieve adequate neutralization. The method can be considered validated if all groups show ▲clearly visible growth visually comparable to that in the control vessel without product within the indicated time period in (71).▲ (USP 1-Dec-2019)

RECOVERY OF INJURED MICROORGANISMS

The validation studies described above use challenge microorganisms that have never been exposed to antimicrobial agents, and thus are not identical to organisms seen in antimicrobial effectiveness testing or when a sterility test is performed on a preserved product. If the use of alternative media is desired, the recovery of injured microorganisms should be addressed in the validation study. This may be done by directly comparing the recovery of each challenge microorganism on the preferred medium and on the alternative medium, after exposure to the product. This exposure should include at least two time periods showing survival of less than 100 cfu/mL, unless the rate of kill of the antimicrobial agent is such that no recovery is possible even if the microorganism is plated within minutes of exposure. This comparison should be performed at least three times. The alternative medium is validated if the recovery seen on that medium is no less than that seen on the preferred medium, within an error of 0.5 log units.

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ESTIMATING THE NUMBER OF COLONY-FORMING UNITS

The accuracy of any estimate of viable cfu is affected by the number plated. As the number of viable cells plated increases, crowding effects decrease the accuracy of the count, reducing the estimate. As the number decreases, random error plays an increasing role in the estimate.

The accepted range for countable colonies on a standard agar plate is between 25 and 250 for most bacteria and *Candida albicans*. This range was established in the food industry for counting coliform bacteria in milk. This range is acceptable for compendial organisms, except for fungi. It is not optimal for counting all environmental isolates. The recommended counting range for *Aspergillus* ▲*brasiliensis*▲ (USP 1-Dec-2019) is between 8 and 80 cfu/plate.

▲▲ (USP 1-Dec-2019)

Lower counting thresholds for the greatest dilution plating in series must be justified. Numbers of colonies on a plate follow the Poisson distribution, so the variance of the mean value equals the mean value of counts. Therefore, as the mean number of cfu per plate becomes lower, the percentage error of the estimate increases (see ▲Table 3). For example,▲ (USP 1-Dec-2019) 3 cfu/plate at the 10⁻¹ dilution provide an estimate of 30 cfu/mL, with an error of 58% of the estimate.

▲Table 3.▲ (USP 1-Dec-2019) Error as a Percentage of Mean for Plate Counts

cfu/Plate	Standard Error	Error as % of Mean
30	5.48	18.3
29	5.39	18.6
28	5.29	18.9
27	5.20	19.2
26	5.10	19.6
25	5.00	20.0
24	4.90	20.4
23	4.80	20.9
22	4.69	21.3
21	4.58	21.8
20	4.47	22.4
19	4.36	22.9
18	4.24	23.6
17	4.12	24.3
16	4.00	25.0
15	3.87	25.8
14	3.74	26.7
13	3.61	27.7
12	3.46	28.9
11	3.32	30.2
10	3.16	31.6

^Table 3.▲ (USP 1-Dec-2019) **Error as a Percentage of Mean for Plate Counts** *(continued)*

cfu/Plate	Standard Error	Error as % of Mean
9	3.00	33.3
8	2.83	35.4
7	2.65	37.8
6	2.45	40.8
5	2.24	44.7
4	2.00	50.0
3	1.73	57.7
2	1.41	70.7
1	1.00	100.0

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