The Most Probable Number Method and Its Uses in Enumeration, Qualification, and Validation

Scott Sutton

"Microbiology Topics" discusses various topics in microbiology of practical use in validation and compliance. We intend this column to be a useful resource for daily work applications.

Reader comments, questions, and suggestions are needed to help us fulfill our objective for this column. Please send your comments and suggestions to column coordinator Scott Sutton at scott.sutton@microbiol.org or journal coordinating editor Susan Haigney at shaigney@advanstar.com.

KEY POINTS

The following key points are discussed:

- The most probable number (MPN) method is useful for estimating quantitative bioburden if plating for colony forming units is not advised.
- This method is described in *United States Pharmacopeia* chapter <61> and by the US Food and Drug Administration in the *Bacteriological Analytical Manual*. Details of the method are discussed.
- The MPN method has direct application in qualification studies for media and for alternate (rapid) microbiological methods. It has also been suggested as a consideration for an alternate method to trend environmental monitoring studies.

INTRODUCTION

The "most probable number" (MPN) method is a useful, if underutilized, tool for the microbiologist. It is part of the harmonized compendial chapter on bacterial enumeration (1) and has been part of the "Microbial Limits Test" chapter in the *United States Pharmacopeia* since the chapter inception in *USP* XVIII (2). The test is a method to estimate the concentration of viable microorganisms in a sample by means of replicate liquid broth growth in ten-fold dilutions and is particularly useful with samples that contain particulate material that interferes with plate count enumeration methods.

The basic concept to the MPN method is similar to the fraction negative method of D-value determination. Nutrient broth will support growth of organisms and turn turbid. The basic pattern of growth vs. no-growth can provide information as it is a reflection of sampling error. For example, if one replicate tube of media receives a dilution of the sample that contains a bacertial cell, the tube will turn turbid. Its neighbor, an "identical" replicate, may not receive any bacteria in its sample due to pipetting or sampling and would not turn turbid. This information is particularly useful at low numbers of organisms. However, this accuracy can be greatly increased by diluting the inoculum and then comparing the recoveries of all tubes in the dilution series. This is the basis of the MPN method

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ABOUT THE AUTHOR

Scott Sutton, Ph.D., is owner and operator of The Microbiology Network (www.microbiol.org), which provides services to microbiology-related user's groups. Dr. Sutton may be reached by e-mail at scott.sutton@microbiol.org.

Table: MPN table for a three-replicate design from FDA's *Bacterial Analytical Manual.*

Positive Tubes					Positive Tubes				
0.1	0.01	0.001	MPN	95% Confidence Range	0.1	0.01	0.001	MPN	95% Confidence Range
0	0	0	<3.0	0-9.5	2	2	0	21	4.5-42
0	0	1	3	0.15-9.6	2	2	1	28	8.7-94
0	1	0	3	0.15-11	2	2	2	35	8.7-94
0	1	1	6.1	1.2-18	2	3	0	29	8.7-94
0	2	0	6.2	1.2-18	2	3	1	36	8.7-94
0	3	0	9.4	3.6-38	3	0	0	23	4.6-94
1	0	0	3.6	0.17-18	3	0	1	38	8.7-110
1	0	1	7.2	1.3-18	3	0	2	64	17-180
1	0	2	11	3.6-38	3	1	0	43	9-180
1	1	0	7.4	1.3-20	3	1	1	75	17-200
1	1	1	11	3.6-38	3	1	2	120	37-420
1	2	0	11	3.6-42	3	1	3	160	40-420
1	2	1	15	4.5-42	3	2	0	93	18-420
1	3	0	16	4.5-42	3	2	1	150	37-420
2	0	0	9.2	1.4-38	3	2	2	210	40-430
2	0	1	14	3.6-42	3	2	3	290	90-1000
2	0	2	20	4.5-42	3	3	0	240	42-1000
2	1	0	15	3.7-42	3	3	1	460	90-2000
2	1	1	20	4.5-42	3	3	2	1100	180-4100
2	1	2	27	8.7-94	3	3	3	>1100	420-4000

(also known and multiple tube, dilution tube, or dilution tube methods). The method offers real opportunities as an enumeration tool. It can also be employed for semi-quantitative estimation of growth-promotion capability of liquid media and in estimation of precision for alternate microbiological methods with a simple modification.

THE METHOD

In the compendial version of the MPN test, the sample to be tested is prepared in 10-fold dilution series, and then 1mL samples of each dilution are inoculated into triplicate broth culture tubes for incubation. As the dilutions increase, the possibility that the broth tubes will fail to be inoculated with any microorganism increases. At some point therefore, very few of the replicate tubes will be inoculated with viable microorganisms.

Following incubation, all tubes are examined for turbidity and the pattern of growth in the tubes is scored against a table of such values (3). The MPN table from the US Food and Drug Administration's *Bacterial Analytical Manual* (BAM) (http://www.fda.gov) is provided above. A typical design uses three replicates with a three-$\log_{10}$ unit dilution series (although varying numbers of replicates and different dilution series may also be used). In this design, if all tubes showed growth, then the results will be noted as 333. If only one tube in each replicate shows growth it would be denoted as 111. The pattern of growth is then read from the table to provide the most probable number and 95% confidence interval. By this, the result of 210 would reflect an MPN of 21, and a result of 322 would be interpreted as an MPN of 210.

Figures 1 and 2 show this in graphic depiction. As the incubated tubes would be read 321, the MPN would be recorded as 150.

The MPN table normally only presents results for three dilutions in sequence (e.g., 10^{-1}, 10^{-2}, 10^{-3}), but the dilution series tested might have been from the 10^{-2} to 10^{-4} tubes (see the FDA BAM discussion on how to select appropriate tubes to read). The worker will need to take the dilution factors in the table and in the actual experiment into account to derive the most probable number from this study. The results of this test should be expressed as "MPN" rather than CFU (colony forming unit) to reflect the capabilities of the method.

Figure 1: Three-tube design for MPN (unincubated).

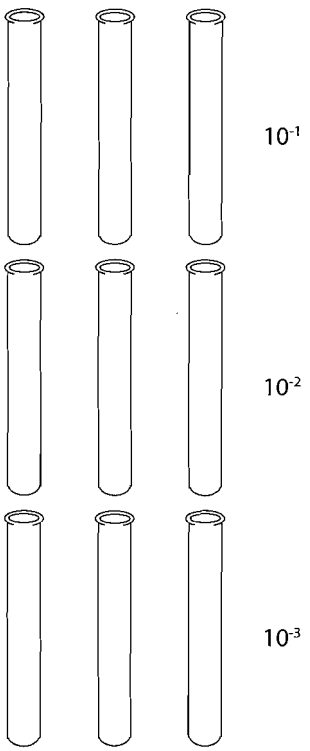
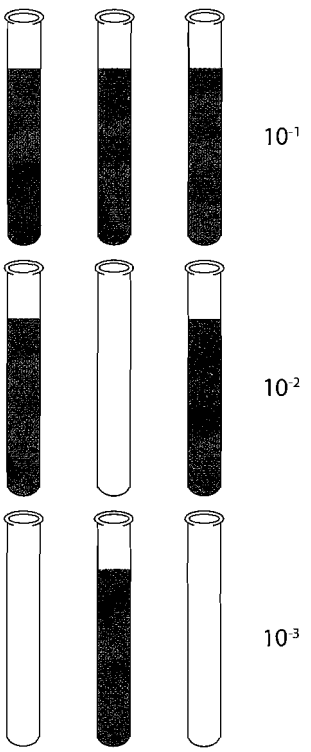


Figure 2: Three-tube design for MPN (incubated).



The method assumes a random distribution of microorganisms in the sample and an accurate dilution of the sample through the dilution series. It also assumes that the microorganisms are separate and do not affect each other (i.e., attract or repel). In addition, it must be assumed that every tube (or plate, etc.) whose inoculum has a single viable organism will result in visible growth.

Although the compendial version utilizes three replicates and a ten-fold dilution series, there is no theoretical reason for these parameters. In fact, it is well known that the accuracy of the method increases dramatically when increasing the number of replicates and decreasing the interval of the dilution series (five-fold or two-fold) (4, 5). The FDA BAM website referenced provides an Excel spreadsheet to assist in creating different MPN tables as needed.

APPLICATIONS OF MPN IN ENVIRONMENTAL MONITORING DATA

Environmental monitoring data is a problem for microbiology. We are urged to "qualify" our control levels and our sample sites. However, we are using a technology (plate count) that is exceedingly imprecise at numbers of CFU below 25. The aseptic core of a modern facility will commonly yield counts of zero, with concern expressed if the count is approaching three. These control levels are in truth of little value despite their popularity in regulatory circles (6-8). One approach suggested to deal with this mismatch between regulatory expectations and plate count capabilities has been to explore the possibility of looking at a frequency distribution models to establish control levels in these areas (9) or incident models (10).

A recent publication by Sun et al. (11) pointed out the possibilities in using MPN methods for evaluation of clean room monitoring data. The basic idea is to use the fundamental statistics as if only a single dilution were being considered. In this approach, the application is not dissimilar to fraction negative studies of biological indicators for sterilization studies. Preliminary studies presented by this group look promising, and this is an approach that could be pursued with existent data for evaluation.

MPN IN QUALIFICATION OF BROTH MEDIA

The compendial chapters of the *USP* on microbial limits tests and sterility tests (revised in 2009) both place great emphasis on media growth promotion studies as a requisite quality control activity. This has been reinforced by the *USP* chapter <1117> (revised in 2010) (12) discussion of the importance of media control in the lab. While

the methods at hand to compare bacterial growth on solid media are quantitative in nature (recovery within 50% or within 70% by CFU), the tools described in the compendia for broth growth promotion are qualitative at best. "Liquid media are suitable if clearly visible growth of the microorganisms comparable to that previously obtained with a previously tested and approved batch of medium occurs" (12).

Weenk (13) reviewed the MPN method in his extensive review of methods used to qualify media and is recommended for its detailed description of how to improve the precision of the method. The basic approach recommended is to approach the MPN method from the opposite direction than that of the bioburden MPN. In the bioburden test, we have a sample with an unknown bioburden and we are trying to deduce the most probable number of cells. In the recommended growth promotion test for liquid media, we have two batches dispensed in tubes. The "sample" is a known inoculum in a known dilution series. The inoculum dilutions are seeded into the two media, and then incubated. If the media exhibit identical growth promoting properties, then the 95% confidence intervals of the two MPN determinations should overlap. In this manner, a (semi)-quantitative growth promotion study may be performed for liquid media.

MPN IN QUALIFICATION OF ALTERNATE (RAPID) MICROBIOLOGICAL METHODS

It should be obvious to the reader that the previous discussion on the use of MPN in growth promotion studies has immediate application for determination of the relative limit of detection for two microbiological methods, for example a "traditional" method and an "alternate" method. This is in fact referenced in *USP* chapter <1223> (14).

The *USP* chapter recommends this approach even for quantitative methods. Although this might at first seem counter-intuitive, the MPN method (when used with a dilution series) can actually be more accurate than plate counts at low numbers. The only modification that needs to be made is to ignore the counts and treat every plate or membrane as a separate "tube"—the MPN method fits right into the experimental design.

SUMMARY

The basic concept for the MPN method is to dilute the sample to such a degree that inocula in the tubes will sometimes (but not always) contain viable organisms. By replicates, and dilution series, this will result in a fairly accurate estimate of the most probable number of cells in the sample. While this method is most commonly used in the pharmaceutical industry for water testing or the compendial bioburden test, it has significant potential to the quality control microbiology lab. These possible applications of MPN include environmental monitoring, media growth promotion studies and aspects of the validation of rapid microbiological methods.

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ARTICLE ACRONYM LISTING

BAM	Bacterial Analytical Manual
CFU	Colony Forming Unit
MPN	Most Probable Number
USP	United States Pharmacopeia