

# The Most Probable Number Method and Its Use in QC Microbiology

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“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](mailto:scott.sutton@microbiol.org) or journal coordinating editor Susan Haigney at [shaigney@advanstar.com](mailto:shaigney@advanstar.com).

## KEY POINTS

The following key points are discussed:

- The most probable number (MPN) method is familiar to quality control (QC) microbiologists as part of the microbial limits tests. Its usefulness goes far beyond this one test, however.
- The theory behind the MPN method is central to the commonly used D-value determination by fraction-negative method, and a variant of this method has been suggested for trending of environmental monitoring data from the aseptic core.
- MPN can be adjusted to provide a sensitive method to determine differences between two qualitative microbiological methods. As such, it can be used as a tool in validation of rapid microbiological methods and for growth promotion testing of broth media.

## 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 (USP)* since the chapter’s 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 10-fold dilutions. It is particularly useful with samples that contain particulate material that interferes with plate count enumeration methods.

The basic assumptions of the MPN method are that bacteria follow Poisson statistics, and that a single viable cell will result in turbidity of the test media under the conditions used. Nutrient broth will support growth of organisms and turn turbid. The basic pattern of growth vs. no-growth can provide information, particularly at low numbers of organisms with large numbers of replicates (3). However, this accuracy can be greatly increased by diluting the inoculum and then comparing the recoveries of all tubes in the dilution series. This then, is the basis of the MPN (also known as multiple tube, dilution tube, or dilution tube methods) method.

The method offers real opportunities as a tool for microbiologists in situations where, for one reason or another, the plate count method is unsuitable. It can also be employed for semi-quantitative estimation of growth-promotion capability of liquid media, in estimation of precision for alternate microbiological methods, and as part of the “fraction negative” method of determining D-values.

#### THE METHOD

In the compendial version of the MPN test, the sample to be tested is prepared in 10-fold dilution series, and then 1 mL 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 (4). The MPN table from the US Food and Drug Administration's *Bacterial Analytical Manual (BAM)* is provided in the Table (4). A typical design uses three replicates with a three- $\log_{10}$  unit dilution series. 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 2,1,0 would reflect an

MPN of 15, and a result of 3,2,2 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 (at least the MPN tables in the harmonized microbial limits tests) will normally only present 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 colony forming unit (CFU) to reflect the capabilities of the method.

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 (attract or repel) (thus are described by the Poisson distribution at low numbers). 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 with increasing the number of replicates and decreasing the interval of the dilution series (five-fold or two-fold) (5, 6). The FDA BAM website referenced provides an Excel spreadsheet to assist in creating different MPN tables as needed (4).

#### MPN AND D-VALUE DETERMINATIONS

Pflug et al. (7) describe the use of the MPN method in D-value determination from a historical perspective. Bigelow and Esty (8) describe the concept of “thermal death time,” which is basically the number of samples of surviving biological indicators at different times. The thermal death time for a temperature was then between the longest heating time yielding a positive unit and the shortest time when all were

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

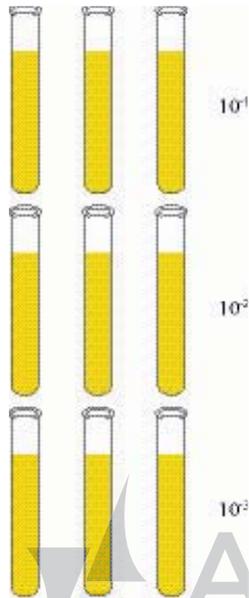
| Positive Tubes |      |       |      |                      | Positive Tubes |      |       |       |                      |
|----------------|------|-------|------|----------------------|----------------|------|-------|-------|----------------------|
| 0.1            | 0.01 | 0.001 | MPN  | 95% Confidence 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             |

negative. In this procedure it was important to use more than one sample. A refinement on this method was proposed by Stumbo et al. (9), who suggested the use of the MPN equation on the surviving units. As there was only one possible dilution, the number of replicates must be representative to provide some precision to the estimate. By varying the heating times we start to develop the survivor curves using this method. There are two other commonly used methods for determining D-values based on this MPN equation—the Stumbo-Murphy-Cochran and the Holcomb-Spearman-Karber methods (9) (which are both a bit complicated to do justice in this overview article. See reference 10 for a review).

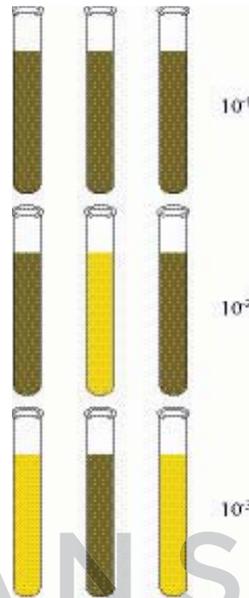
**APPLICATIONS OF MPN IN ENVIRONMENTAL MONITORING DATA**

Environmental monitoring data are 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. However, 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 (11, 12, 13). 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

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



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



frequency distribution models to establish control levels in these areas (14) or incident models (15).

A recent publication by Sun et al. (16) 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 on microbial limits tests and sterility tests both place great emphasis on media growth promotion studies as a requisite quality control activity. This has been reinforced by the revised 2010 *USP* chapter <1117> (17) 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 with-

in 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" (17).

Weenk (18) reviewed different methods of demonstrating equivalence between two media. His study looked at the different methods for both solid and liquid media, but we will look only at the methods for liquid. He looked at the following to compare nutrient broth performance:

- Length of lag phase in each media from identical inocula
- Growth rate in each media
- Endpoint determination
  - Total biomass at stationary phase
  - Agar inoculation (number of CFU at stationary phase)
- Adding agar to 15 g/l nutrient broth, then using quantitative enumeration techniques
- MPN.

He found the length of lag phase to be widely variable, the growth rate determination for each media prohibitively labor-intensive. The endpoint determinations were found to be too imprecise and labor intensive. One wonders what he would have concluded from the harmonized method of inoculating with <100 CFU and approving it if growth occurs.

The MPN method was recommended by Weenk (18) to qualify media and is recommended for its accuracy, cost, and flexibility. The basic approach recommended is to approach the MPN method from the opposite direction than that of the compendial 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.

To set this up, one design might be to prepare twelve 10 ml tubes of each medium. The inocula (however many microorganisms used) are then prepared to an approximate concentration of 50, 5, 0.5, and 0.05 CFU/tube (this has to be approximate only). Each dilution is then added to three tubes of each media, and the media incubated. After incubation, the MPN is separately deduced for each media—hopefully the confidence intervals will overlap.

### 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 (e.g., a “traditional” method and an “alternate” method). This is in fact referenced in *USP* chapter <1223> (19).

The author first realized this during preparation to teach at the 2004 PDA-TRI course “Rapid Microbiological Methods” (October 2004). Using the method described for liquid media, qualitative methods could easily be compared for “Limit of Detection.” This method eventually appeared in the finalized chapter (20).

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, especially if five replicates are used and the dilution series is less than 10-fold (the 10-fold dilution series is common only because of tradition and the availability of standard tables—there is no theoretical obstacle to a 5-fold dilution series, or 3-fold, or a 2-fold, or others). 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 personal products, medical device, and pharmaceutical QC microbiology labs for water testing or the compendial bioburden test, it has significant potential for other applications. These possible applications of MPN include D-value determination, environmental monitoring, media growth promotion studies, and aspects of the validation of rapid microbiological methods.

### REFERENCES

1. *USP*, Chapter <61> “Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests,” *United States Pharmacopeia* 32, vol. 1, pp 71-75, 2009a.
2. *USP*, “Microbial Limit Tests,” *USP XVIII*, p. 846, 1970.
3. Halvorson, H.O. and N.R. Ziegler, “Application of Statistics to Problems In Bacteriology: II A Consideration of the Ac-

- curacy of Dilution Data Using a Single Dilution," *J Bacteriol.* 26(4):331-339, 1933.
4. FDA, *Bacterial Analytical Manual*, Appendix 2, <http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm109656.htm>  
BAM Excel spreadsheet can be downloaded from the [www.fda.gov](http://www.fda.gov) site at <http://www.fda.gov/downloads/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/UCM164329.zip>
  5. Eisenhart, C and W Perry, "Statistical Methods and Control In Bacteriology," *Bacteriol Rev.* 7:57-137, 1943.
  6. Cochran, W., "Estimation of Bacterial Densities by Means of the Most Probable Number," *Biometrics.* 6:105-116, 1950.
  7. Pflug, IJ, et al., "Principles of the Thermal Destruction of Microorganisms," *Disinfection, Sterilization and Preservation*, 5th Ed. SS Block (ed), Lippincott Williams & Wilkins pp79-134, 2001.
  8. Bigelow, W.D. and JR Esty, "The Thermal Death Point In Relation to Time of Typical Thermophilic Organisms," *J Infect Dis* 27:602-617, 1920.
  9. Stumbo, C.R., JR Murphy and J Cochran, "Nature of Thermal Death Time Curves for P.A. 3679 and Clostridium Botulinum," *Food Technol.*, pp. 321-326, 1950.
  10. Takahashi, M., et al., "Comparison of D-Value Accuracy Among Limited Spearman-Karber Procedure, Stumbo-Murphy Cochran Procedure (SMCP) and Limited SMCP," *PDA J Pharm Sci Tech.* 49(5):220-225, 1995.
  11. Hussong, D and RE Madsen, "Analysis of Environmental Microbiology Data From Cleanroom Samples," *Pharm Technol Aseptic Proc*:10-15, 2004.
  12. Farrington, JK., "Environmental Monitoring in Pharmaceutical Manufacturing-A Product Risk Issue," *Amer Pharm Rev* 8(4):26-30, 2005.
  13. Agalloco, J. and Akers, J., "The 'Myth' Called Sterility," *Pharm Tech, Bioprocess Sterile Manufact*:s44-s45, 2010.
  14. Caputo, RA and A Huffman, "Environmental Monitoring: Data Trending Using a Frequency Model," *PDA J Pharm Sci Tech.* 58(5):254-260, 2004.
  15. USP, Chapter <1116> "Microbiological Control and Monitoring Environments Used for the Manufacture of Healthcare Products," *Pharm Forum* 33(3), 2007.
  16. Sun, X et al., "The Expanded Application of Most Probable Number to the Quantitative Evaluation of Extremely Low Microbial Count," *PDA J Pharm Sci Tech* 60(2):124-134, 2006.
  17. USP, Chapter <1117> "Microbiological Best Laboratory Practices," *USP 33 Reissue Second Supplement*, pp. R-1100-R-1105, 2010.
  18. Weenk, G.H., "Microbiological Assessment of Culture Media: Comparison and Statistical Evaluation of Methods," *Intl J Food Microbiol.* 17:159-181, 1992.
  19. USP, Chapter <1223> "Validation of Alternative Microbiological Methods," *USP 32* vol. 1 pp730-3, 2009b.
  20. USP, Chapter <1223> "Validation of Alternative Microbiological Methods," *USP 29 Suppl 2* pp. 3807-3810, 2006.

GXP

#### ARTICLE ACRONYM LISTING

|            |                                 |
|------------|---------------------------------|
| <b>BAM</b> | Bacterial Analytical Manual     |
| <b>CFU</b> | Colony Forming Unit             |
| <b>FDA</b> | US Food and Drug Administration |
| <b>MPN</b> | Most Probable Number            |
| <b>QC</b>  | Quality Control                 |
| <b>USP</b> | United States Pharmacopeia      |

#### ABOUT THE AUTHOR

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