

Determination of Inoculum for Microbiological Testing

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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 or journal managing editor Susan Haigney at shaigney@advanstar.com.

IMPORTANT POINTS

The following key points are discussed:

- Quality control microbiology tests require controlled levels of inocula and require fresh preparations of cells for those inocula
- The concentration of cells in a suspension can be estimated by optical density, but this must be confirmed by plate count
- The optical density readings against cell mass are specific to the microorganism species
- The qualification of these readings must be confirmed after major maintenance to the benchtop spectrophotometer (e.g., after replacement of the bulb).

INTRODUCTION

The ability to quickly estimate the number of viable cells in a microbial suspension is important in several laboratory applications. It is a critical component of method suitability tests where the usual expectation is to demonstrate recovery of low numbers of a set of challenge organisms in the presence of the product. This is used for sterility tests, microbial limits tests, and the antimicrobial effectiveness test (AET). The AET also uses a high inoculum as the challenge system for the test. The most direct method for determining the microbial count is to plate the microbial suspension, and then infer the number of cells from the number of colony-forming units (itself, of course, only an approximation). However, this method requires a significant amount of time (minimum of 18 hours) and is not suitable for these methods, as they require “fresh” suspensions.

DETERMINATION OF INOCULUM FOR THE AET

The compendial antimicrobial efficacy test (AET) requires inoculation of the product with microorganisms to a final concentration of approximately 10^6 CFU/mL. Although this seems to be a minor point, it does serve to illustrate some of the inherent difficulties in microbiological testing and the need for experienced and academically trained microbiologists to head the laboratory.

The *European Pharmacopoeia* (1) instruction on preparing the inoculum for the AET states:

“To harvest the...cultures, use a sterile suspending fluid...Add sufficient suspending fluid to reduce the microbial count to about 10^8 micro-organisms per milliliter...Remove immediately a suitable sample from each suspension and determine the number of colony-forming units per milliliter in each suspension by plate count or membrane filtration (2.6.12). This value serves to determine the inoculum and the baseline to use in the test. The suspensions shall be used immediately.”

There are, of course, two problems with these instructions. The first is that the technician is instructed to use an inoculum of about 10^8 microorganisms per milliliter and then instructed to determine this by plate count. Colony-forming units (CFU) and cells (i.e., micro-organisms and spores) are different measures. This will inevitably lead to difficulties as the unfortunate lab worker cannot guarantee the number of cells in the suspension, only the number of CFU found. However, we can accept the scientific inaccuracy, as the numbers will generally work out. The more serious problem is the instruction to use the plate count CFU for determination of the inoculum for the test, and that the suspension shall be used immediately. This, quite frankly, cannot be done. If you use the suspension immediately, the plate counts are unavailable; if you use the plate counts to set the inoculum, then the suspension is at least a day old.

Contrast these instructions with those in the *United States Pharmacopeia (USP)* (2) for the same exercise:

“To harvest the...cultures, use sterile saline...Add sufficient...to obtain a microbial count of about 1×10^8 CFU per mL...[Note: The estimate of inoculum concentration may be performed by turbidimetric measurements for the challenge organisms. Refrigerate the suspension if it is not used within 2 hours].

Determine the number of CFU per mL in each suspension...to confirm the initial CFU per mL estimate. This value serves to calibrate the size of the inoculum used in the test.”

These USP instructions have the advantage of being physically possible to perform, an advantage that cannot be underrated. However, the turbidimetric measure of the cells is also only an approximation of CFU. Thus the instruction to confirm the numbers (after the test is underway) with the plate count is an important control on the test.

This article explores the turbidimetric approximation for cell numbers, the important controls on the process, and the potential pitfalls to the process.

Light scattering techniques to monitor the concentration of pure cultures have the enormous advantages of being rapid and nondestructive.

However, they do not measure cell numbers nor do they measure CFU. Light scattering is most closely related to the dry weight of the cells.

Light is passed through the suspension of microorganisms, and all light that is not absorbed is re-radiated. There is a significant amount of physics involved in this, and those interested are referred to optical treatises, particularly those discussing Huygens' Principle (a good choice is *Light Scattering by Small Particles* by H C Van De Hulst). For our purposes, it is enough to say that light passing through a suspension of microorganisms is scattered, and the amount of scatter is an indication of the biomass present in the suspension. In visible light, this appears “milky” or “cloudy” to the eye (3). It follows from this that if the concentration of scattering particles becomes high, then multiple scattering events become possible.

TABLE: McFarland turbidity standards.

McFarland Scale	CFU (x10 ⁶ /mL)	1% BaCl ₂ /1% H ₂ SO ₄ (mL)
0.5	<300	0.05/9.95
1	300	0.1/9.9
2	600	0.2/9.8
3	900	0.3/9.7
4	1200	0.4/9.6
5	1500	0.5/9.5
6	1800	0.6/9.4
7	2100	0.7/9.3
8	2400	0.8/9.2
9	2700	0.9/9.1
10	3000	1.0/9.0

METHODS

McFarland Turbidity Standards

McFarland standards can be used to visually approximate the concentration of cells in a suspension. The McFarland Scale represents specific concentrations of CFU/mL and is designed to be used for estimating concentrations of gram negative bacteria such as *E. coli*. Note that this estimate becomes uncertain with organisms outside the normal usage, as different species of bacteria differ in size and mass, as do yeast and mold. Use of this method for various organisms would require calibration and validation.

McFarland standards are generally labeled 0.5 through 10 and filled with suspensions of barium salts. Latex bead suspensions are also available, which extend the shelf life of the material. The standards may be made in the lab by preparing a 1% solution of anhydrous BaCl₂ and a 1% solution of H₂SO₄ by mixing them in the proportions listed in the Table. They should be stored in the dark, in a tightly sealed container at 20-25°C, and should be stable for approximately six months (4).

The advantage of the use of these standards is that no incubation time or equipment is needed to estimate bacterial numbers. The disadvantage is that there is some subjectivity involved in interpreting the turbidity, and that the numbers are valid

only for those microorganisms similar to *E. coli*. In addition, the values are not in the appropriate range for the AET inoculum and further dilutions may be required.

Spectrophotometer

The spectrophotometer measures turbidity directly. The best case (i.e., most sensitive) would be to have a narrow slit and a small detector so that only the light scattered in the forward direction would be seen by the detector. This instrument would give larger apparent absorption readings than other instruments (see Figure).

As should be obvious, each spectrophotometer used must be independently calibrated for use in estimating microbial concentrations. Not only is the apparent absorption affected by the width of the instrument's slit, the condition of the filter, and the size and condition of the detector, but also each time the lamp is changed the calibration needs to be repeated as different bulbs may vary in total output.

The correlation of absorption to dry weight is very good for dilute suspensions of bacteria (5), and this relationship seems to hold regardless of cell size (although the relationship of absorption to CFU does not). However, in more concentrated suspensions, this correlation (absorption to dry weight) no longer holds.

The linear range of absorption to estimated CFU is of limited scope. For this reason, the calibration study must demonstrate the linear range of the absorbance versus CFU values and the relevant values.

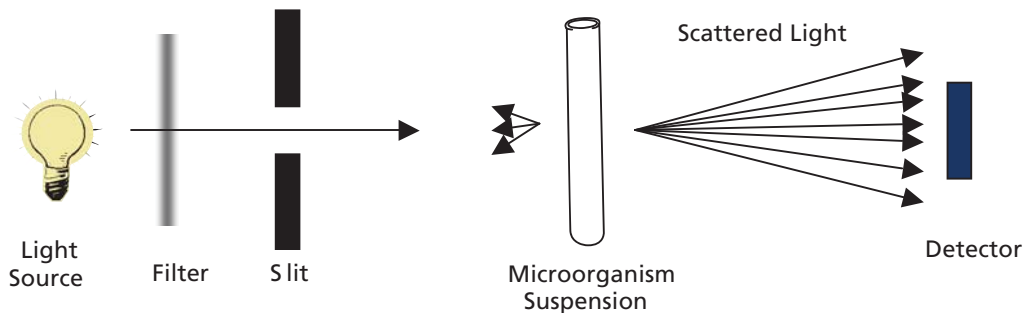
Procedure

As there are a variety of different instruments, there cannot be one single procedure. In general, the spectrophotometer can be set at a wavelength of 420–550 nm. This wavelength must be standardized.

It is important to have the cells in known physiological state of growth. That is to say, as the cell size varies with phase of growth (i.e., lag, log, stationary), the approximate relationship between absorbance and CFU will also vary. A recommended practice might be to pass a single well-isolated colony twice on overnight cultures from the refrigerated stock, and harvest the rapidly growing culture from the

Figure:

Spectrophotometer components.



second passage. This also will serve to minimize a source of variability for the AET (6).

A second source of concern might be the cuvette used for the measurement—care must be taken to maintain the correct orientation of the cuvette, and to protect it from damage that could affect the passage of light. Finally, it is necessary to blank the spectrophotometer (i.e., adjust the absorbance reading to zero) using a standard, either water or the suspending fluid, and maintain this practice.

Calibration

It must be stressed that this calibration should be done for all organisms. The size of the organism, any associated pigments, the preparation of the suspension, and other factors all influence the readings. This calibration study should also be rechecked after changing the bulb on the light source and should be reevaluated throughout the life of the light bulb.

The calibration itself is simple to perform. Prepare a concentrated solution of the organism, grown under the conditions that will be used for the test. Make a series of dilutions to cover the range of absorption measurements of interest; five to eight dilutions are recommended. Immediately take the spectrophotometer readings in sequence, and then take a confirmatory reading of the first in series to confirm that no growth has occurred. The dilutions are then immediately plated for viable count (serial dilution of the suspensions will be necessary). Graph the relationship between the absorbance and the

CFU/mL after the plate counts are available and use values in the linear range of this graph. This linear range may require a wide dilution series to identify, and similar raw dilutions (from the nutrient broth) might not yield samples in the linear range.

As there are several factors that can affect this curve (e.g., quality of lamp output, size of slit, condition of filter, condition of detector, microorganism characteristic, etc.), this calibration should be confirmed when the conditions of the assay change. In fact, all spectrophotometer estimates should be treated as just that and confirmed by plate count in parallel with the test.

TROUBLESHOOTING

There are several potential areas where the method might provide erroneous results.

Mix-n-Match

The first of these potential mistakes involves indiscriminate use of information from the Internet. There has been some discussion of this topic on the *Pharmaceutical Microbiology Forum Email List* (PM-FList) (7), and it is clear that many workers fail to understand how instrument-dependent these optical density curves are. Each one must be checked for the specific organism and instrument. Just because a reading of 0.14 gave an acceptable number of CFU/mL in one facility for a specific species of microorganism does not mean that it will be acceptable anywhere else or for other microorganisms.

Where Did it Come From?

The QC test is concerned about number of CFU. Optical density (OD) is an indirect measure of cell mass. Different species, even if all other aspects of the system are identical, will have different optical density readings for similar CFU/mL concentration. You cannot use similar OD numbers for different species without confirmation. Some cells (e.g., *C. albicans*) might have CFU/mL ten-fold lower than other commonly used microorganisms at the same OD reading.

Is it Clumping?

This method obviously assumes a fairly homogeneous suspension of cells. This is sometimes not the case. For example, *B. subtilis* is a naturally competent (able to transfer DNA among cells) microorganism and in the competent state aggregates in media. This is not conducive to good OD readings. *A. brasiliensis* spores also tend to clump, or even to get hung up in hyphal mats during preparation. Sometimes the inclusion of small amounts of polysorbate into the suspending buffer and filtration through sterile glass wool is helpful to generating a more homogenous suspension of this organism.

OD Drift

The lamp in the spectrophotometer will age. It is useful (and recommended) that a log book of daily OD readings against the measured CFU be kept to assist in determining when the calibration of the curve should be repeated or when the lamp should be replaced.

CONCLUSIONS

The use of optical density to estimate CFU in a suspension is possible if basic precautions are taken. It is important to control the following:

- The physiological state of the organism
- The species of the organisms
- The nature and condition of the equipment.

Despite the inherent inaccuracy of the method, if the procedure is adequately controlled and calibrated, the estimation of microbial numbers by optical density (either by McFarland Standards or spectrophotometrically) is sufficiently accurate for use in preparing inocula for QC testing. This method offers the advantages of being rapid, low cost, and non-destructive.

REFERENCES

1. EP, "5.1.3 Efficacy of Antimicrobial Preservation," *European Pharmacopoeia* 6.6 pp, 5129-5130.
2. USP, <51> Antimicrobial Effectiveness Testing, *United States Pharmacopoeia*, 2011.
3. Koch, AL. "Growth Measurement," *Methods for General and Molecular Bacteriology* Gerhardt, P et al. (ed) American Society for Microbiology, Washington, DC. p. 248-277, 1994.
4. Smibert, RM and NR Kreig, "Phenotypic Characterization" Section 25.4.9, *Methods for General and Molecular Bacteriology*, Gerhardt, P et al. (ed) American Society for Microbiology, Washington, DC. p. 607-654, 1994.
5. Koch, AL., "Turbidity Measurements of Bacterial Cultures in Some Available Commercial Instruments," *Anal Biochem* 38:252-259, 1970.
6. Gilbert, P. et al. Inocula for Antimicrobial Sensitivity Testing: a Critical Review. *J Antimicrob Chemother.* 20:147-154, 1987.
7. www.microbiologyforum.org. **GXP**

ARTICLE ACRONYM LISTING

- AET** Antimicrobial Efficacy Test
CFU Colony Forming Units
OD Optical Density
QC Quality Control
USP United States Pharmacopeia

ABOUT THE AUTHOR

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