

Measurement of Microbial Cells by Optical Density

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:

- Quality control (QC) 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 bench top spectrophotometer (e.g., after replacement of the bulb).

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 10^8 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 *Pharmacopeia Europe* (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 (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:

For more Author information, go to gxpandjvt.com/bios

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.

"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 turbidometric 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 will explore the turbidometric approximation for cell numbers, the important controls on the process, and the potential pitfalls to the process.

THEORY

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.

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

Table: McFarland turbidity standards.

McFarland Scale	CFU ($\times 10^6$ /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

mold. Use of this method 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 now 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 mixed 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 so further dilutions may be required.

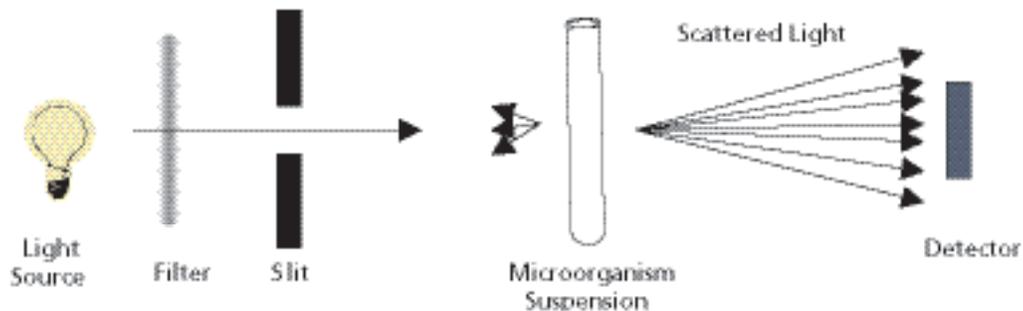
Spectrophotometer

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. The apparent absorption is affected by the width of the instrument's slit, the condition of the filter, and the size and condition of the detector. Whenever 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

Figure: Spectrophotometer.



(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 vs 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 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; 5 to 8 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.

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.

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. The method offers the overwhelming advantages of being rapid, low cost, and non-destructive.

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ARTICLE ACRONYM LISTING

AET	Antimicrobial Efficacy Test
CFU	Colony Forming Unit
QC	Quality Control
USP	United States Pharmacopeia



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