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Automated, Digital Colony Counting: Qualification and Data Integrity

By Tim Sandle Mar 21, 2018 12:34 pm PDT

Introduction

Many microbiological tests depend on an accurate count of bacterial and fungal colonies. The process of enumeration is, for the busy laboratory, a relatively slow, tedious task. Moreover, it is not always reliable and open to variations (‘error’) in terms of accurate counting and the counts recorded between different technicians. The counting of colonies relies upon the ability of the technician to 'see' colonies. The extent to which colonies are clear and countable is a product of microbial growth, in addition to the quality of the growth media and the incubation conditions to which the plate has been subject to (time and temperature are the key parameters) (1). Idealized incubation will lead to the formation of colonies that are readily discernible by their improved size, shape, distribution and contrast. This is, in practice, not always the case. Moreover, the method by which the sample was captured can have a bearing on the result. Here, for example, many environmental monitoring techniques can affect the likelihood or otherwise of microbial survival, as with the desiccation effect that can occur with settle plates or the shearing forces associated with air samplers. The application of automated colony counters can help to speed up the process and to improve counting precision provided a reliable and qualified instrument is used.

Hence many laboratory managers have given attention to automated, digital devices to streamline the plate counting processes. Such devices have been commonplace to the clinical laboratory for some time; although adoption within pharmaceutical laboratories has been slower. Recent regulatory interest in data integrity and the economic pressures driving the ‘lean’ laboratory, with a focus on cost control, improving sample throughput, and reviewing whether each sample tested adds value or produces meaningful information (2), may account for the growing interest from the pharmaceutical sector.

The better examples of automated colony counters are devices that are not affected by the inoculation method, shape or size of the colony. In particular, an effective automated colony counter will have good sensitivity for the detection of smaller colonies, particularly in low contrast media and the ability to capture counts and images using digital technology (3). Many will also have the ability to transfer data to Laboratory Information Management Systems (LIMS). This paper considers the selection and adoption of automated, digital capture colony counters within the pharmaceutical microbiology laboratory. The focus is on how such devices can be qualified (or validated) and how data integrity concerns can be addressed, provided that the former has been appropriately planned and executed.

Problem Statement #1: Colony Counting

Before considering the application of automated colony counters it is prudent to consider the two intertwined problems with counting microorganisms on agar. The first relates to counter colonies per se; the second is the issue of data integrity.

Looking at colony counting first, the colony forming unit (CFU) is the primary means of assessing microbial levels on solid media, whether this is an assessment of the presence or absence of a particular microbial species; an attempt to enumerate the total count; or with an analysis of non-zero events (as might be undertaken for aseptic processing). The CFU is the product of growth-based agar plate count methods intended to estimates the microbial population within a sample. The generation of a CFU utilizes microorganisms' capability to replicate under the applied medium, temperature, and time conditions. These factors of time and temperature, while of great interest, fall outside the scope of this paper (1).

Many text books erroneously portray the colony forming unit as a single organism. This derives partly from the way bacteria reproduce (multiply) via binary fission under the controlled conditions (the assumption that one bacteria is present on or in agar and this organism then multiplies to form a sufficient number of clones to form an observable colony). However, the reason for the 'colony forming unit' in place of 'number of bacteria counted' in relation to growth observed on agar was to remove this uncertainty as to whether the colony reflected one or more organisms. Reasons why a CFU may represent more than one organism include the fact that many bacteria do not occur naturally as one cell. For example, some bacteria grow in chains (e.g. Streptococcus) or clumps (e.g. Staphylococcus); the consequence of poor mixing of a sample before plating out, where cells stick together or become bound to the sample. Bacillus species, for example, are notorious for clumping; and the method of collection, as with a settle plate where a CFU develops from where a microbial carrying particle has been deposited and that particle may have been host to more than one organism.

Colonies can be difficult to count for several reasons (4):

a) The act of colony counting is not only repetitious it can lead to errors and thus problems of data integrity.

b) In low count assays minor counting errors will have significant effects.

c) A related error is when numbers of CFUs on a plate can lead to false results due to overcrowding of bacteria.

d) Indistinguishable colony overlap (i.e., masking).

e) Assessing colonies near the plate periphery.

f) Increased density of collected culturable microorganisms.

Some of the concerns are illustrated by the following images:

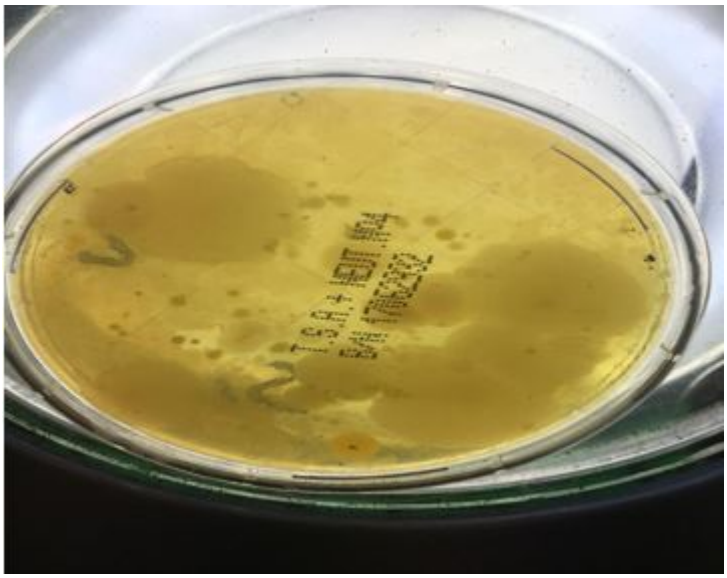

Figure 1: The problem of mixed colonies and merging colonies (Image: Tim Sandle)

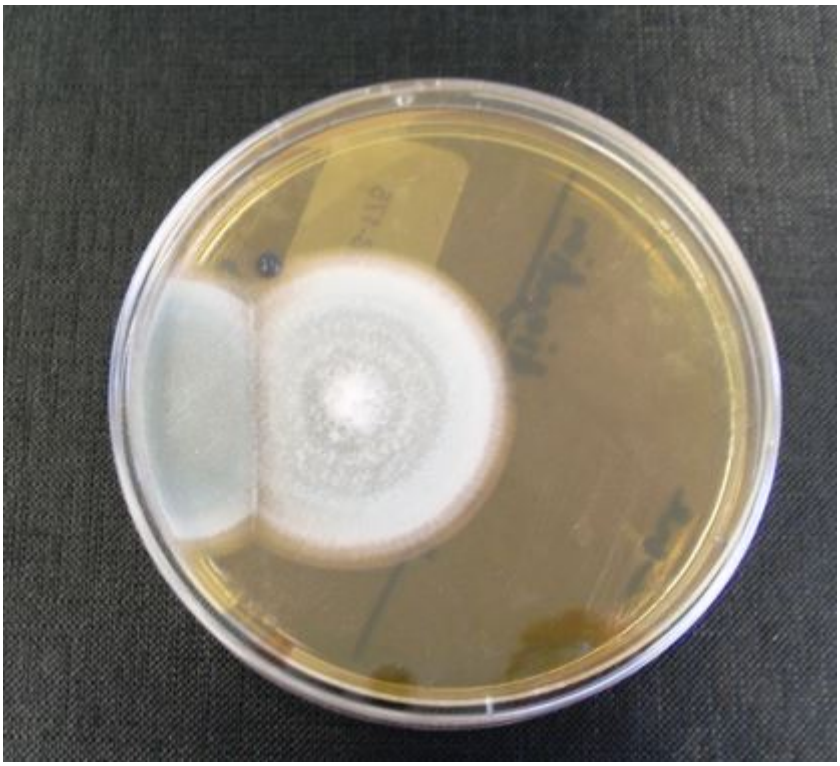

Figure 2: The problem presented by merging fungal colonies (Image: Tim Sandle)

Figure 3: Concern with a large number of colonies towards the periphery of the plate (Image: Tim Sandle)

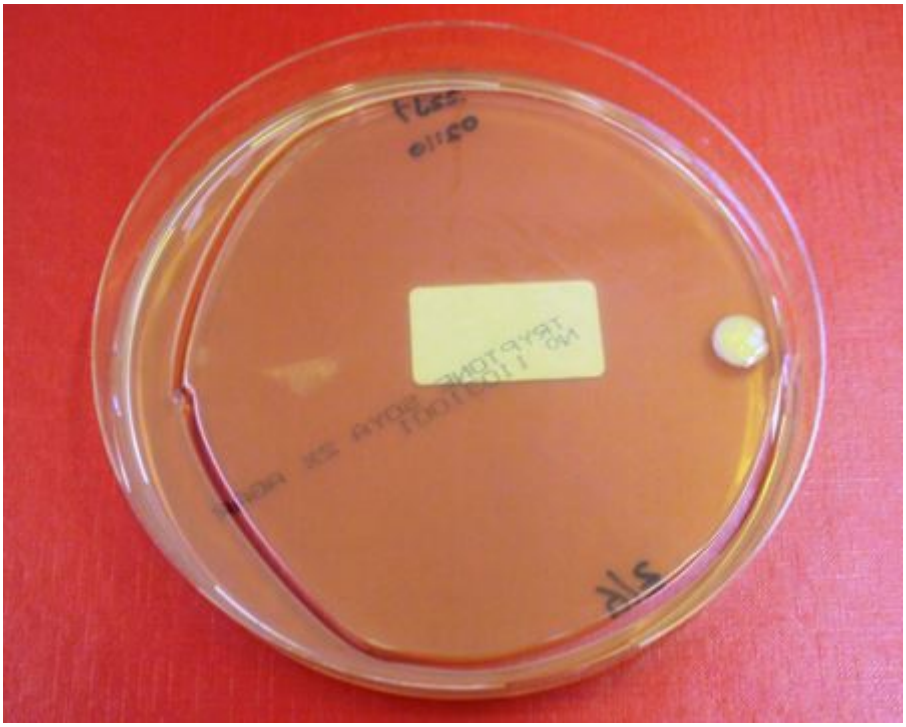

Figure 4: Cracked plates can cause problems, for result validity (Image: Tim Sandle)

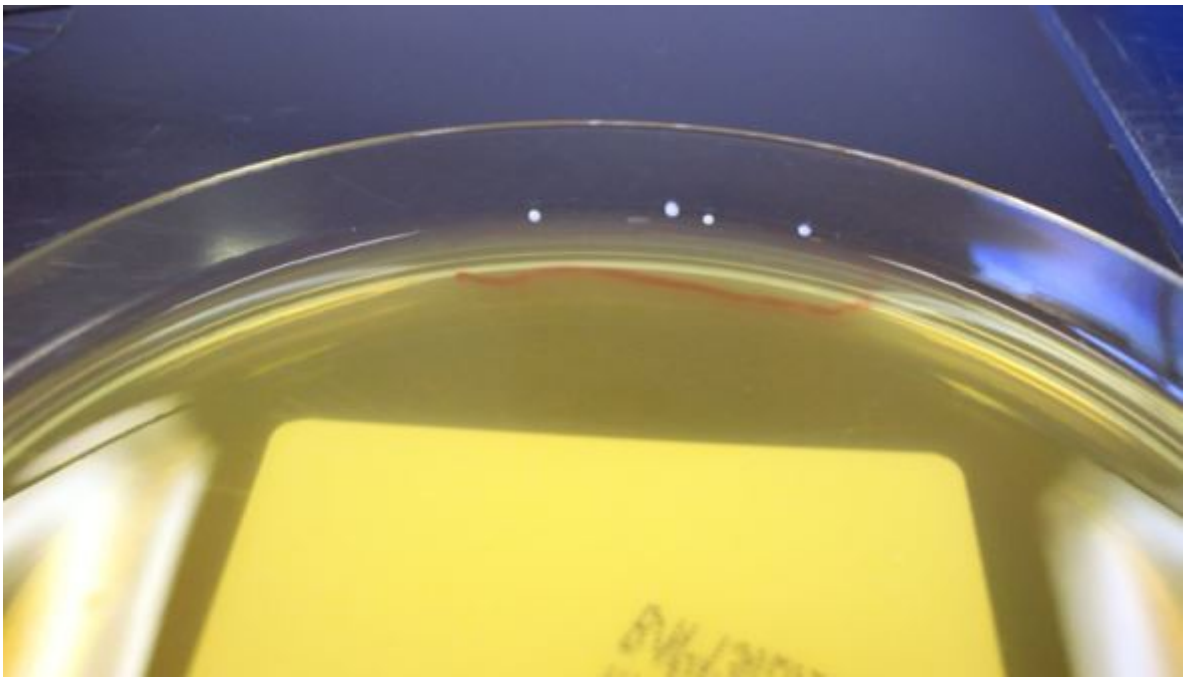

Figure 5: Unusual growth of colonies off the agar, on the side of the Petri-dish (Image: Tim Sandle).?

With 'b' and 'c' above, the countable range varies according to the test method (for a pour plate, for example, this is generally placed within 25 to 250 CFU or 30 to 300 range; with membrane filtration this generally accepted as being between 20 to 80). The countable range is important since it avoids an overcrowding error occurs from individual colonies inhibiting the formation of other colonies nearby.

Sutton has provided some excellent overviews of the plate counting problem (5, 6). Factors affecting the upper countable range include colony size and behavior (possible swarming, as the case with organisms like Proteus mirablis and some Bacillus species, such as Bacillus cereus), as well as the surface area of the plate. At the lower end can be added the difference between limit of quantification (which is what arguably really matters, and with a standard 9 centimeter plate is 25 CFU) and limit of detection (which many setters of 'specifications' assume can be reliably recovered, often going down as a low as 1 CFU). Error arises with the lower limit because the CFU's follow Poisson distribution where the error of the estimate is the square root of the mean.

These are some of the reasons why automatic colony counters are considered as an option to reduce plate counting errors.

Problem Statement #2: Data Integrity

Data integrity refers to maintaining and assuring the accuracy and consistency of data over its entire life-cycle, and is a critical aspect to the design, implementation and usage of any system which stores, processes, or retrieves data. Data integrity is a key regulatory concern (7, 8) and guidance documents have been produced by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Moreover, data integrity in relation to the microbiology laboratory is featured in several FDA warning letters, especially in relation to sample handling and reading.

The concept of data integrity applies to computerized systems, paper documentation, and other means of capturing data (down to the level of reading a microbiological environmental monitoring sample) (9). Thus, data integrity is relatively broad in scope and it can have widely different meanings in different situations. Microbiology laboratories handle voluminous amounts of data, although unlike analytical laboratories the data is more often an even mix of quantitative and qualitative data. Despite the current high profile, the subject of data integrity in the pharmaceutical microbiology laboratory has not been afforded very much attention in terms of regulatory guidance or in terms of active discussion through articles penned by those

working in the pharmaceuticals or healthcare sectors.

An important area for data integrity in microbiology is with culture media. Plate reading and counting is an area of data integrity concern. Plates can be incorrectly read by a technician; misinterpreted; or incorrectly counted. The manual process can be partly addressed by training staff appropriately in plate reading, including training in typical colonial morphology and how to interpret colonies that have merged together (confluent growth) or where spreading, by a motile organism has occurred. Understanding such limitations is the basis of good microbiology laboratory practices (10).

Improvements can be introduced through reading plates against a white light source and by using a colony counter, fitted with a magnifying glass. Consideration can also be given to second checks or random checks performed by a supervisor. In addition, any sample at alert or action level should be subject to a confirmatory check as part of local out-of-limits / specification procedures. Some of these are discussed further by this author in a Journal of GxP Compliance paper on data integrity for the pharmaceutical microbiology laboratory (11).

A different solution is provided by automated colony counters that read the plate in place of a technician and capture the result electronically. As a note of caution, however, such colony counters can create their own data integrity concerns if they are not properly qualified.

Automated Colony Counters

Microbiological procedures rely on an accurate count of bacterial colonies and cells. Automated colony counters are used to estimate a liquid culture's density of microorganisms by counting individual colonies on an agar plate, slide, mini gel, or Petri dish (12). Instruments accommodate various sizes and formats, including units that can be used to view plates up to 300 × 300 mm. Some counters can be optimized for use with ultraviolet light illumination, white light, fluorescent, and green fluorescent protein colonies (as might be the case with the pigment produced by Pseudomonas aeruginosa) (13). The counting can be accomplished manually, often with touch pressure and a digital counter, or can be semi- or fully automatic. There are many different commercial systems available, and each has a proprietary algorithm for the image analysis and interpretation (14); based on image smoothing processes and intensity gradient field discrimination (15). Systems are addressed in general terms in this paper and no specific system is mentioned directly.

In terms of functionality, the best automated colony counters should offer (16 - 21):

- Standardized and accurate results. Accuracy is important since colony counting can be affected by numerous parameters related to the physical properties of the colony: size, shape, contrast, and overlapping colonies. To achieve this requires automatic colony separation (for when colonies are positioned close to each other).
- Ability to count colonies within appropriate parameters (such down to 50 microns and measure zones accurately to 0.5 millimeters, within detection limits of 0.1 millimeters).
- Good optical response performance (sufficient control of the background noise, contrast, resolution, etc.) of the image acquisition tools.
- Effective image resolution, file size/data management, sample lighting, and instrument uniformity.
- Ability to visualize white light and fluorescent colonies.
- The ability to differentiate between chromatic and achromatic images and thus deal with both color and clear media.
- Able to separate aggregated colonies.
- The ability to count the entire plate or sectors of the plate.
- Results obtained within one second per plate.
- The display of real-time full-color on-screen images.
- Zoom function for looking at smaller colonies.
- Software to allow for data collection and analysis. Data should ideally be transferrable to a Laboratory Information Management System (LIMS).

The essential elements of automated, digital colony counters include a circular dark field illuminator and a camera with a resolution of 3.3 megapixels or higher (many systems have cameras of higher quality); software with appropriate algorithms; an automated plate holder (with a toolbox to enable communication between the software and the image analyzer). With the software algorithm many work on the basis of a Bayes classifier. This is a simple probabilistic classifier used to study the geometric properties such as ratio between major and minor axis of the group are used to verify the number of colonies contained in the group (22).

Some types of counters offer greater functionality. For example, some can provide data on the size or shape of the colonies and measure zones of inhibition via software. More sophisticated software can also provide color and size differentiation (allowing microbiologists to distinguish colonies of different hues) (23). Other systems have the functionality to count and calculate concentrations of spiral-plated colonies (24).

Earlier detection is another potential that automated colony counters can provide. This relates to the quality of the imaging system. Some scientific-grade cameras, for instance, can detect colonies down to 43 microns (a level that is on the edge of being visible to the eye).

Factors Affecting Automated Colony Counter Performance

There are several factors which can affect the success of an automated colony counter. Some of these are similar to the errors that relate to manual colony counting and some relate to computerized image analysis. For example:

a) Colony size;

b) Colony number;

c) Overcrowding and conglomeration;

d) Degree of convexity;

e) Colony brightness;

f) Colony shape;

g) Colony texture.

The better systems being marketed will have overcome most of these challenges.

Validation of Automated Colony Counters

Validation of automated colony counters is important and necessary for implementation into the laboratory. To ensure the validity of their data, microbiologists need to establish that their automated colony counting method is as accurate as a precise manual count before they implement any new process into their workflow. Some validation aspects will be available as a package from the manufacturer (typically a mix of statistical methods for simulating cell colony images, as well as manual counting, to quantify the proprietary algorithm accuracy) (25). However, the purchasing laboratory will also need to qualify the instrument within the laboratory setting.

There are different approaches that can be taken for the laboratory validation. The acceptance criteria should be decided in advanced. This would normally be with reference to the manufacturer's won recommendation. To seek 100% accuracy would, perhaps, be unrealistic (especially based on the errors abound with manual plate counting. The acceptance criteria should be based on an acceptance range (the system may slightly undercount and over-count) for both individual plates and for the set of plates assessed. A range of 95-105%

might be appropriate. To assess the difference between manually counted and automatic colony counts, an appropriate statistical method should be used, such as a two-tailed t-test for paired samples with a 95% confidence level.

An example of a validation approach:

a) Compare automated colony counting with routine manual counting. With manual counting the counted CFU should be marked with a pen on the plate cover to discriminate counted from uncounted colonies.

b) Ensure that plates with a range of different counts are used (up to what is considered the maximum counting range).

c) Ensure that plates with pure and mixed colonies are used, including bacteria and fungi (it would be difficult to specify in advance the number of different colony types to include). With the selection of pure cultures it makes sense to obtain these from a recognized culture collection, to allow for standardization and to avoid any concerns about colony purity (provided that good aseptic technique has been followed). The cultures selected here can either relate to those typically used with compendial tests or those likely to challenge the system. For appropriate challenges, consider colonies where the physical properties range from smooth to hairy, from bright to opaque, and from high to low convexities (26).

d) Ensure each type of agar used in the laboratory is assessed, especially agars of different colors or hues.

e) For an initial test of the system use pure cultures of known organisms. If the system fails to detect satisfactorily, there is little point in going forwards.

f) The more robust test is using plates from microbial testing. This should include plates from tests where colonies are within the agar (such as from a bioburden test performed using the pour plate method) and where colonies are on top of the agar (such as a settle plate used for environmental monitoring).

g) In terms of the numbers of plates to assess, a minimum of 100 for both pure cultures and 'real life' mixed samples would be appropriate.

h) To ensure robustness, at least three different technicians should take part.

Following on from the confirmation of counting; the appropriate capture of the data should be verified in relation to the inability to manipulate the data; arching; retrieval and transmission to another computerized system. Furthermore, an automated system should give each sample a unique identifier; this should also be checked. Where an image is taken and stored as a photograph, a confirmation that the image is correct should also be made. Some of these data integrity issues are assessed below.

Validation is not always successful. Weaknesses can occur where there are mixed colonies or, due to inhomogeneity of the agar thickness; meaning that discrimination is not possible for all areas of the plate. A further weakness is where confluent growth occurs. The light also needs to be right; here white light dark field illumination has been shown to work well, however a blue dark field illumination provides the best discrimination of all (27). A further problem that can arise is by the presence of debris and other unwanted material, either embedded within or on the surface of the agar. Some of these weaknesses can be overcome through re-assessing the samples or making adjustments to the instrument. Where significant weaknesses cannot be addressed and thus the acceptance criteria is not met, then the automated colony counter is probably not suitable for use.

Assessing Computerized Systems

With computerized systems, like automated colony counters, there are various guidance documents available. Guidance has also been produced by the FDA (2016) and the U.K. MHRA (2015), together with other regulatory agencies. These documents provide useful advice, for example, stating that systems should

be designed in a way that encourages compliance with the principles of data integrity. Some examples of this could include (28):

- Access to clocks for recording timed events.
- Accessibility of laboratory records at locations where activities take place so that ad hoc data recording and later transcription to official records is not necessary.
- User access rights which prevent (or audit trail) data amendments.
- Automated data capture or printers attached to colony counters.
- Proximity of printers to relevant activities.
- Access to raw data for staff performing data checking activities.

Both regulatory agencies place an importance, in terms of computerized systems, on access and password control. Full use should be made of access controls to ensure that people have access only to functionality that is appropriate for their job role, and that actions are attributable to a specific individual. Furthermore, systems should be in place to ensure data back-up.

Auditing Computerized Systems

When carrying out internal audits, especially with respect to computerized systems, five key data integrity questions are (29):

- Is electronic data available?
- Is electronic data reviewed?
- Is meta data (audit trails) reviewed regularly?
- Is there clear segregation of duties?
- Has the system been validated for its intended use?

This can be helpful in pre-preparing for a regulatory inspection. On this matter, common regulatory inspection areas include:

- Data processing and review, accuracy checks
- Potential for data manipulation and deletion
- Repeat testing / replicate data
- Date / time stamp manipulation
- Criteria used to invalidate data
- Data transfer to systems - Checks that data are not altered in value and/or meaning
- Level of checking should be statistically sound
- Security of the system and user access levels – appropriate segregation of duties
- Electronic signatures – use of individual and generic passwords
- Regular back-ups of all relevant data should be done. Integrity and accuracy of backup data and the ability to restore the data should be checked during validation and monitored periodically.
- Archived data should be checked for accessibility, readability and integrity.
- Audit trails.
- Change management, including changes to a part of the system may pose a risk due to interdependencies.

There are additional, system specific areas. However, covering these basic points provides the foundation for conducting a data integrity review of automated colony counters.

Open Source Software

For laboratories that do not have stringent data integrity considerations, such as with universities or for classroom teaching, open source software provides an alternative means for colony counting through photographic analysis. One example is OpenCFU, which is a free software that can facilitate (and render more reproducible) the enumeration of colony forming unit (CFU) (30). The user can run the program on a

computer and input pictures of plated bacterial colonies (or other cells), and obtain a result. An alternative open source platform is CellProfiler, which is used to automatically identify and measure a variety of biological objects in images (31).

Summary

This paper has examined the use of automated colony counters and the means to assess and qualify them in the laboratory setting. The paper has come at this subject with a focus on error reduction and taking into consideration data integrity issues.

If applied properly, automated colony counters can provide a faster and more reproducible means to capture microbial test results. A key practical advantage is with automatically transferring results to a computerized system Central to this the validation, and microbiologists need to be confident that the automated colony counting technology is as accurate (or better) than a manual counting method and that the data is secure and cannot be manipulated. With this is place, greater accuracy of counts can be obtained together with data security.

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Tim Sandle
Tim Sandle, Ph.D., is the head of the microbiology department at Bio Products Laboratory Limited. Dr. Sandle is, additionally, a visiting tutor at the School of Pharmacy, Manchester University and a committee member of...
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