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Evaluation of Accuracy Limits of Countable Colony-forming Units on Agar Plates

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Abstract:

Objectives: Accurate colony counts are an essential component of many microbiology research projects and clinical laboratory processes. The suggested range of accuracy of CFUs extends from 30 to 300 (Standard Methods for the Examination of Water and Wastewater). This recommendation dates to 1907, and fails to adequately address the numerous sources of inter- and intra-variability. Without more detailed analysis it is difficult to estimate the sample size and number of replicates necessary to ensure accurate results. The purpose of this study was to determine the validity of accuracy limits for quantifying CFUs on agar plates.

Methods: *Escherichia coli* (ATCC 25922) and *Staphylococcus epidermidis* (ATCC 12228) were used to prepare series of four organism densities ranging from approximately 40-500 CFU, on three different days. On each day, each of the 4 densities for both organisms was plated on SBA and viable organisms were counted following incubation. An average of the margins of error obtained over the 3 days of testing was used to determine the reproducibility of agar plate counts, and to estimate the optimum number of replicate plates (sample size) required for each organism at each concentration.

Results: Margins of error for both organisms were greatest with suspensions yielding approximately 40 CFU, and lowest for suspensions yielding 300 and 500 CFU. Nine replicate plates were required for a suspension of *S. epidermidis* yielding 40 CFU to achieve the same margin of error as obtained with 3 replicate plates at concentrations yielding 100-300 CFU. Seven replicates plates were required for a suspension of *E. coli* yielding 40 and 100 CFU to achieve similar margins of error to those obtained with 4 replicate plates at concentrations yielding 300 CFU, and 3 replicate plates at concentrations yielding 500 CFU.

Discussion: We found that the greater the concentration (300 and 500 CFU), the fewer replicate plates necessary to reliably estimate organism concentrations. The lower the organism density (40 CFU), the more plates necessary to reliably estimate CFUs. Contrary to the recommendations described in Standard Methods for the Examination of Water and Wastewater, CFU of 500 were reliably reproducible. For greatest accuracy, experiments should be conducted so as to assure that colony counts are in the range of 300-500.

Introduction:

Accurate colony counts are an essential component of many microbiology research projects and clinical laboratory processes. The universally accepted range of accuracy for colony counts from agar plates is 30-300, and is based on recommendations approved by the Standard Methods Committee and published in the most current edition of Standard Methods for the Examination of Water and Wastewater. This recommendation dates back to 1915.

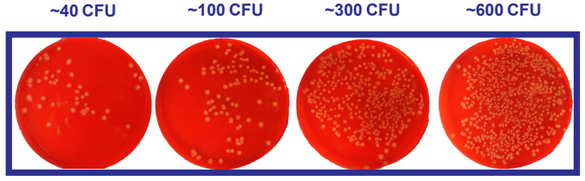
Studies supporting the 30-300 range have not adequately addressed accuracy. Colony counting is inherently associated with a previously undetermined degree of variability. The purpose of this study was to determine the accuracy and reproducibility of published limits for countable CFU on agar plates.

Methods and Materials:

Suspensions of *Escherichia coli* (ATCC 25922) and *Staphylococcus epidermidis* (ATCC 12228) were prepared in triplicate in 0.85% saline (pH 7.0) to equal a 0.5 McFarland Standard (~1.5 x 10^8 CFU/ml)[2] in a nephalometer.

Staphylococcus epidermidis

Escherichia coli



Serial dilutions were performed to produce final suspensions that assured colony counts in the range of 40, 100, 300 and 500. On each of the three test days, 100 ul of each of the 4 organism densities was plated on each of 25 SBA plates. Colonies were counted under a stereo-microscope following incubation for 24 hours (*E. coli*) and 48 hours (*S. epidermidis*) in ambient air at 35°C.

An average of the colony counts obtained was multiplied by its dilution factor to determine the initial concentration in CFU/mL. The observed concentration mean was then compared to the published expected concentration of 1.5 x 10^8 CFU/mL to determine which colony count provided results closest to the expected mean.

An average of the margins of error obtained over the 3 days of testing was used to determine the reproducibility of agar plate counts, and to estimate the optimum number of replicate plates (sample size) required for each organism at each concentration.

Statistical Analysis

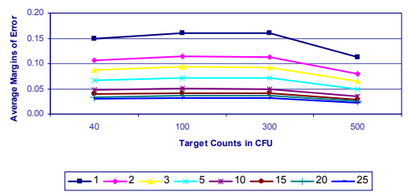
Accuracy was measured by comparing actual plate counts of the number of colony-forming units (CFU) to the expected number of CFU. To determine whether there was any difference in accuracy between the different target counts (40, 100, 300, 500), a Randomized Complete block design was used with Day as the blocking variable. Precision was measured by the half-length of a 95% confidence interval.

Results:

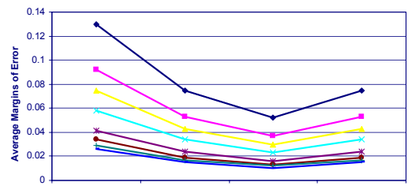
Reproducibility

CFU versus number of replicate plates (based on average margins of error)

Escherichia coli



Staphylococcus epidermidis



Number of Replicate Plates versus Target CFU Count

S. epidermidis ATCC #12228 *E. coli* ATCC #25922

CFU Mean	37 (20-64)	79 (42-118)	239 (146-356)	466 (248-656)	CFU Mean	48 (28-69)	131 (76-190)	370 (218-493)	620 (343-806)	CFU Mean
#PLATES					#PLATES					#PLATES
1	0.13	0.075	0.052	0.075	1	0.150	0.161	0.160	0.112	1
2	0.092	0.053	0.053	0.053	2	0.106	0.114	0.113	0.079	2
3	0.075	0.043	0.03	0.043	3	0.087	0.093	0.092	0.065	3
5	0.058	0.034	0.023	0.034	5	0.067	0.072	0.071	0.050	5
10	0.041	0.024	0.016	0.024	10	0.047	0.051	0.050	0.035	10
15	0.034	0.019	0.013	0.019	15	0.039	0.042	0.041	0.029	15
20	0.029	0.017	0.012	0.017	20	0.034	0.036	0.036	0.025	20
25	0.026	0.015	0.01	0.015	25	0.030	0.032	0.032	0.022	25

* Margins of error are presented in CFU x 10^8/mL

Reproducibility

Margins of error were greatest for *S. epidermidis* with suspensions yielding approximately 40 CFU. As expected, as the number of replicate plates increased, the margin of error was reduced. Ten plates were required for a suspension of *S. epidermidis* yielding a mean of 37 CFU to achieve a similar margin of error as two plates with mean colony counts of 466.

Five plates were required for a suspension of *E. coli* yielding means of 48, 131 and 370 CFU, to achieve similar margins of error obtained with three replicate plates at concentrations yielding 620 CFU.

Results (cont'd):

Accuracy

Analysis using two-way analysis of variance indicated a significant day to day variability in the inoculum density [day effect ($p < 0.0001$)]. As a result, the average number of colonies at each dilution varied considerably around our target on days 1,2 and 3 by up to three-fold.

For *E. coli*, the calculated CFU/ml from plates with approximately 100 CFU most closely approximated the density expected based upon nephalometry (0.5X 10^8 vs. 1.5 X 10^8) ($p = 0.0146$).

For *S. epidermidis*, the calculated CFU/ml from plates with approximately 40 and 500 CFU most closely approximated the density expected for *S. aureus* based upon nephalometry (3.67 x 10^7 and 3.72 x 10^7, respectively, vs. 8.2 x 10^7).

Discussion:

There are two prinicipal considerations when doing colony counts. They are inextricably linked.

1. How accurately do the results reflect the actual number of viable organisms in the suspension?

2. How precise are the counts and how many plates need to be counted and averaged to reliably determine the answer, while at the same time limiting the number of plates for practicality?

The choice of which colony density to enumerate, especially when serial dilutions have been prepared, may influence the results.

Our data suggest that suspensions of *E. coli* and *S. epidermidis* prepared using a nephalometer may contain fewer organisms than previously thought. Plates with approximately 100 colonies were closest to the expected for *E. coli*, but were still only 1/3 of the expected density. Plates with approximately 40 and 500 colonies were closest to the expected for *S. epidermidis* but were still only 1/3 of the expected density.

There is an obvious appeal in counting 40 rather than 500 CFU/plate; therefore, increasing the number of replicate plates may be preferable to counting more densely populated plates. It should also be noted that reliable assessment of 500 CFU depends on the size of the colonies, whether or not they spread or coalesce during growth, and whether or not suspensions contain mixed populations of cells, where one species forming smaller CFU may be obscured by larger colonies, especially as the inoculum becomes more dense. In these cases, 500 CFU may be too many to count.

Conclusion:

If colonies are mostly discrete, our data suggests that counting larger numbers may give more reliable results and fewer plates need to be counted.

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