Estimating MU for microbiological plate count – using intermediate reproducibility duplicates method

Before looking into the calculation aspect of this subject, let's get a few important definitions in right perspective:

1. Precision

Precision can be defined as the closeness in agreement between results tested independently under stipulated conditions. It covers three possible levels: repeatability r, within-lab repeatability $s_{R'}$ (intermediate reproducibility), and between-lab reproducibility s_R. Precision is usually expressed as the variance, standard deviation, coefficient of variation or relative standard deviation RSD of a series of test results.

The expected precision of culture-based microbial count methods is typically derived mathematically based on the assumption that bacteria are distributed randomly in a well-mixed sample.

The bacterial growth follows a Poisson distribution which is a model with a discrete distribution, involving the probability of counts of occurrences in a given sample. It is used to describe random phenomena in which the probability is small but constant. It is interesting to note that the variance (σ^2) of a Poisson distribution is equal to the mean (μ) and the Expected Value (λ), i.e. $\sigma^2 = \mu = \lambda$. One will then infer that the standard uncertainty of a bacterial count is then the squared root of the mean value, i.e. $\sqrt{(\mu)}$! Actually it is not that simple.

2. Repeatability, *r*

Repeatability can be defined as the closeness in agreement between measured quantity values obtained by replicate measurements of the same analyte carried out under the *same conditions* of measurement (i.e. same analyst, equipment and reagents) *over a short interval of time*. Repeatability is also termed within-run precision.

The spread of results is measured by the repeatability standard deviation, s_r and the relationship between r and s_r is $r=2.8s_r$.

3. Intermediate repeatability (intermediate reproducibility), $s_{R'}$

Intermediate repeatability can be defined as the closeness in agreement between measured quantity values obtained *within* a laboratory out of a set of conditions that includes the same measurement procedure and replicate measurements on the same or similar samples over an extended period of time by different analysts and possibly different equipment.

This intermediate repeatability $s_{R'}$ is expected to be larger than s_r for the same test procedure as it covers more variations. It is indeed a better assessment of the laboratory's performance on the measurement procedure concerned.

4. Reproducibility, R

Reproducibility can be defined as the closeness of the agreement between the results of successive measurements of the same analyte under the same conditions of measurement but in different laboratories.

The spread of mean results reported by the participating laboratories is measured by the inter-laboratory standard deviation of reproducibility, s_R and the relationship between r and s_R is $r=2.8s_R$.

5. Replicate

A replicate is a counterpart of another, usually referring to a test sample or a measurement. It is the general case for which ***duplicate***, consisting of two samples or measurements, is the special case.

The use of replicate samples data is to demonstrate closeness of agreement between results of successive measurement of the same sample analyte under the same condition of measurement. A very important criterion is that the sample for replicate analysis must be *as homogeneous as possible* to minimize any uncertainty contributed by sub-sampling for examination.

6. Laboratory control sample (LCS)

Laboratory control sample (LCS) is one of the many quality control samples used to identify the source of analytical error and to ensure the laboratory's analytical performance is reliable. The LCS for a microbiology laboratory can be a certified reference material or a laboratory fortified blank with a known quantity of microorganism inoculated, also referred to as spike blank. The concentrations of organisms in LCS are usually within the range of normal analysis of the matrix concerned.

Uncertainty estimation method by reproducibility duplicates for LCS

The following discussions are based on the A2LA guide G108 – *Guidelines for estimating uncertainty for microbiological counting methods*, with a crossed reference to ISO/TS19036: *Microbiology of foods and animal feeding stuffs – Guidelines for the estimation of measurement uncertainty for quantitative determinations.*

In the course of routine analysis with QC protocols in place, an accredited laboratory is to run LCS of which matrix is representative of the samples analyzed by the laboratory regularly in parallel with the batch of samples. And, for any microbiological count examination, duplicate or replicate analysis is always a norm.

Hence, over time, a wealth of LCS analysis results can be collated after going through all of the steps of the test method, set up over different days in duplicate, by different analysts using different equipment (such as balances, incubators, pipettors, *etc.*) and possibly different batches of media and reagents. It is good to know how to make full use of these QC data which lead us to intermediate reproducibility after some simple statistical manipulations.

The adoption of a holistic top down measurement uncertainty (MU) approach with the "intermediate reproducibility replicates" to estimate uncertainty for the same type of sample matrix is therefore a good way to evaluate how various uncertainty contributors affect the routine results under different within-laboratory conditions.

Limitations of this method

a. The ISO/TS19036 suggests that this step-by-step approach in the overall performance of the analytical process is not applicable to: the analysis of very low levels of microorganisms, where the results of plate count are less than 10 CFU's because these results may be well below the limit of quantitation (LOQ).

b. Does enumeration using a most probable number (MPN) technique also require an estimation of its uncertainty? Many learned organizations have different opinions on this question. Since MPN is already a statistical probability estimation, many are of the opinion that it does not need such MU evaluation anymore.

c. In microbiological count experiment, a serial dilution decimally with a diluent sterile broth starting from 10^{-1} is usually carried out. Its influence on the result uncertainty cannot be over emphasized. The following types of replicates are not suitable for this approach as they might lead to significantly underestimate the combined uncertainty:

- ✧ Plate replicates: dilutions on one matrix are carried out by one analyst, and duplicate dilution plates of the single control sample are made
- ✧ True replicates: the original sample is split and diluted in two separate and independent series of dilutions by one analyst, and *only* one set of plates is prepared from each sample.

General calculation method

As microbiological data do not normally conform to a "normal" probability distribution, a mathematical transformation is required prior to statistical analysis. For most practical purposes, a $\log_{10}$ transformation is sufficed to "normalize" the data.

To recap our high school mathematics, $log_{10}(10) = 1$, $log_{10}(100) = 2$, and so $antilog(2) = 10^2$ or 100.

The calculation steps follow the very basic statistical principle for standard deviation of a set of duplicated data, a and b. Assuming there are n sets of duplicated data:

$a_1, b_1;$
$a_2, b_2;$
$a_3, b_3;$
……;
$a_i, b_i;$
……;
a_n, b_n

where n = the total number of pairs of duplicates. The differences between replicates are:

$D_1 = a_1 - b_1$
$D_2 = a_2 - b_2$
$D_3 = a_3 - b_3$
……..
$D_i = a_i - b_i$
……..
$D_n = a_n - b_n$

When all the D's are squared, summed up and divided by 2, we have the mean of sum of squares (MSS):

$$MSS = \frac{\sum_{i-1}^{n} D_i^2}{2} \qquad \text{Eq [1]}$$

The combined standard uncertainty expressed as combined standard deviation therefore is:

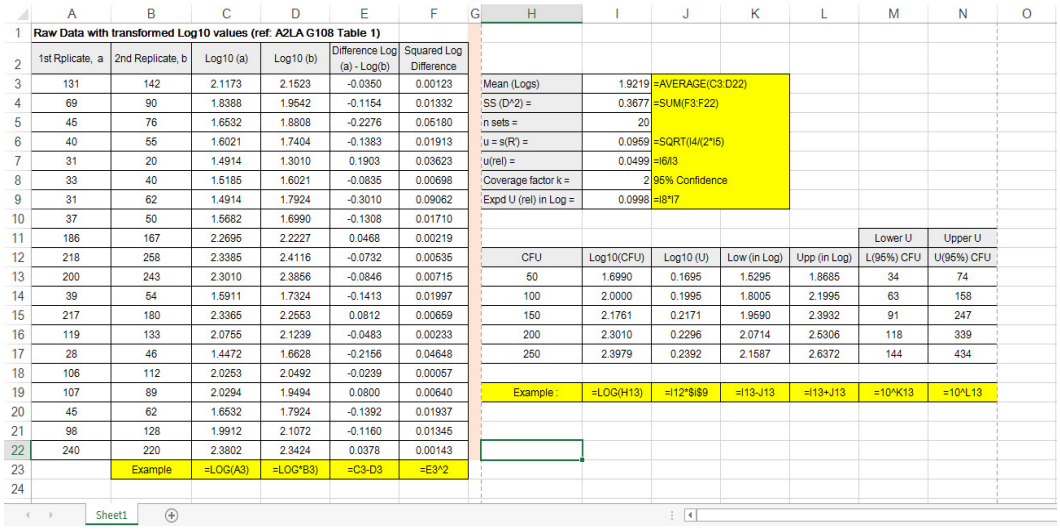
$$u_c = s_{R'} = \sqrt{\frac{MSS}{n}} = \sqrt{\frac{\sum_{i=1}^{n} D_i^2}{2n}} \qquad \text{Eq [2]}$$

The relative standard uncertainty is then

$$u_{rel} = \frac{s_{R'}}{\bar{x}} \qquad \text{Eq [3]}$$

where $\bar{x}$ is the mean value of the n sets of paired data.

Image 1 below shows the 20 CFU raw replicate data generated under intermediate reproducibility conditions and the related calculations made in MS Excel spreadsheet. The data were reproduced from the A2LA G108 example (Table 1). The calculation formulae are shown in the yellow boxes.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	**Raw Data with transformed Log10 values (ref: A2LA G108 Table 1)**														
2	1st Rplicate, a	2nd Replicate, b	Log10 (a)	Log10 (b)	Difference Log (a) - Log(b)	Squared Log Difference									
3	131	142	2.1173	2.1523	-0.0350	0.00123		Mean (Logs)	1.9219	=AVERAGE(C3:D22)					
4	69	90	1.8388	1.9542	-0.1154	0.01332		SS (D^2) =	0.3677	=SUM(F3:F22)					
5	45	76	1.6532	1.8808	-0.2276	0.05180		n sets =	20						
6	40	55	1.6021	1.7404	-0.1383	0.01913		u = s(R') =	0.0959	=SQRT(I4/(2*I5)					
7	31	20	1.4914	1.3010	0.1903	0.03623		u(rel) =	0.0499	=I6/I3					
8	33	40	1.5185	1.6021	-0.0835	0.00698		Coverage factor k =	2	95% Confidence					
9	31	62	1.4914	1.7924	-0.3010	0.09062		Expd U (rel) in Log =	0.0998	=I8*I7					
10	37	50	1.5682	1.6990	-0.1308	0.01710									
11	186	167	2.2695	2.2227	0.0468	0.00219							Lower U	Upper U	
12	218	258	2.3385	2.4116	-0.0732	0.00535		CFU	Log10(CFU)	Log10 (U)	Low (in Log)	Upp (in Log)	L(95%) CFU	U(95%) CFU	
13	200	243	2.3010	2.3856	-0.0846	0.00715		50	1.6990	0.1695	1.5295	1.8685	34	74	
14	39	54	1.5911	1.7324	-0.1413	0.01997		100	2.0000	0.1995	1.8005	2.1995	63	158	
15	217	180	2.3365	2.2553	0.0812	0.00659		150	2.1761	0.2171	1.9590	2.3932	91	247	
16	119	133	2.0755	2.1239	-0.0483	0.00233		200	2.3010	0.2296	2.0714	2.5306	118	339	
17	28	46	1.4472	1.6628	-0.2156	0.04648		250	2.3979	0.2392	2.1587	2.6372	144	434	
18	106	112	2.0253	2.0492	-0.0239	0.00057									
19	107	89	2.0294	1.9494	0.0800	0.00640		Example :	=LOG(H13)	=I12*I9	=I13-J13	=I13+J13	=10^K13	=10^L13	
20	45	62	1.6532	1.7924	-0.1392	0.01937									
21	98	128	1.9912	2.1072	-0.1160	0.01345									
22	240	220	2.3802	2.3424	0.0378	0.00143									
23		Example	=LOG(A3)	=LOG*B3)	=C3-D3	=E3^2									
24															

Sheet1

From the above example, we can report that for a result of 150 *CFU*, the uncertainty interval is 91 to 247 *CFU*.

The above MS Excel spreadsheet can be made as a template for estimating MU of microbiological plate count experiment and the LCS data can be updated as and when available. The results of estimated uncertainty are thus dynamic and current. This is indeed the big advantage of using the holistic top down approach for MU estimation.

In conclusion, it is noted that the sources of uncertainty reflected by this intermediate reproducibility method with LCS cover the analytical random error, counting error, dilutions, environment, equipment and also the performance of analyst concerned.