Rapid Microbiological In-Silico Enumeration (ISE)

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Abstract- *This work focuses on a novel, indirect enumeration method, In-Silico Enumeration (ISE). CO_2 concentrations are detected and converted into TOU-Transmittance of Optical Units via the Soleris Rapid Microbiological Detection System with Fusion Software. These TOU values, when plotted on a TOU vs. Time graph, generate a Sigmoidal curve characteristic of microbial growth curves. Using TOU values recorded at specified time points the ISE method generates cfu/g microbial counts in test samples comparable to those achieved from direct plating procedures. Log difference values for these assays are within ± 0.5 log differences of plate counts with an R^2 value >0.91. This work also addresses the accuracy, robustness, limitations, and precision of this novel test method. This work further demonstrates how the ISE method generates counts in real-time and without the need of plating procedures. Sample cfu/g counts can be generated in as little as twenty-four hours for aerobic microbes and forty-eight hours for yeast/mold microorganisms. This reported ISE Method represents a novel procedure for the enumeration of aerobic microorganisms and is an alternative testing procedure capable of replacing plates used for microbial enumerations.*

Importance: There are two general ways to enumerate microbes, directly and indirectly. The direct approach requires slow and tedious procedures performed by trained analysts. Indirect methods quantify microbes using test methods other than direct colony observation. The time too results is faster, but they come with inherent challenges. Counts from these methods are usually general estimates at best or only qualitative at worst. One notable example of an indirect method technology is the Biolumix/Soleris Rapid Microbiology Detection System (System). Currently, the System is limited to qualitative/semi-quantitative microbiology, and due to its limited enumeration capabilities inadvertently raises the level of false positives needing enumeration tests by direct plating techniques, the very procedures it was created to replace! In short, the System wasn't designed to enumerate. The ISE method was created to eliminate this and allow the System to enumerate aerobic microbes with Direct Plate Method accuracy with Indirect Method speed.

Introduction: Direct methods get their name from directly observing and counting the microbial growth present on the test plates. These results, reported as colony forming units per gram of test sample (cfu/g) are the gold standard for traditional microbiology enumerations. However, the use of a cfu unit for the accurate enumeration of a sample's bioburden should be understood to be an estimate and not an exact count. Exact counts in nature are not possible due to fluctuations in growth conditions, i.e. temperature, nutrient presence, etc. Further, individual species of microbes typically do not grow independently of each other but rather as complex communities. Finally, the time and resources needed for direct test methods are items

that a fast-paced pharmaceutical/nutraceutical industry doesn't always have in large quantities. Thus, quicker time-to-result, indirect test methods were developed.

Indirect methods may also use units other than the cfu to report growth. Thus, a correlation between the cfu and the indirect method reporting unit should be established if the indirect method wishes to achieve comparable results to those of the traditional Direct Method. Simply stated, industry needs the speed of indirect methods and the enumeration accuracy of direct methods.

One of the most widely recognized, indirect method technologies, the Biolumix/Soleris Rapid Microbiology Detection System (System), has been serving the pharmaceutical/nutraceutical industry for well over a decade. Its purpose is to decrease the time to microbial enumerations by eliminating the need for slower, direct plating methods. Other instruments, based on different technological platforms, have also been created for the same purpose.

Currently, the System is limited to qualitative/semi-quantitative microbiology. The analyst dilutes the test sample to a given cfu/g specification, enters this value as the test specification for the System, and performs the assay. The System will then report a less than value of the desired specification if there is no growth. Microbial growth, if present, is indicated by a positive rate of change in the growth curve during the assay. The System flags this rate of change as a presumptive assay failure and by default assumes that the tested sample exceeds this test specification resulting in a semi-quantitative report of the sample's bioburden content.

This growth curve is the detection of the light transmittance, measured in Optical Units (TOU), that passes through a green tinted agar plug- sensor- at the bottom of a test vial. CO_2, a metabolic by-product of microbial growth, diffuses through this sensor turning it yellow. The TOU increases as more light passes through the sensor as it becomes more yellow due to the rising amounts of CO_2 being generated by the increasing numbers of viable microbes in the test vial. This detected positive increase in the TOU is flagged by the System as a presumptive failure that will require confirmation testing using direct plating methods. By default, this technology inadvertently raises the level of false positives needing confirmation tests by direct plating techniques, the very technology it was created to replace. In short, the System wasn't designed to enumerate, but what if it could?

A 2022 research article reported general CO_2 vs cfu/mL growth curves in heating oil (1). These exponential growth curves linked cfu/mL to milligram concentrations of CO_2. The In-silico Enumeration (ISE) method is based on those reported growth curves. The ISE method was optimized to utilize microgram levels of CO_2 and normalized to the TOU generated by the System. After all this, it is now possible to get direct method accurate cfu/g counts in samples in an indirect Rapid Microbiology Detection System!

Materials and Methods: Five separate growth curves were generated using the challenge microorganisms listed in Table 1. These five challenge microorganisms were diluted to approximately 1-3 cfu/mL. 1mL of these diluted challenge microbes were transferred to Soleris Total Viable Count (TVC) vials for the *S. aureus, E. coli*, and *B. subtilis* microbes and Total Yeast/Mold Vials (YM-109) for *C. albicans* and *A. brasiliensis*. These inoculated vials were then placed into the System. The bacteria at 35°C and the yeast/mold at 28°C. All vials completed a normal growth cycle as per the System's recommended Total Aerobic Count and Total

Yeast/Mold test procedures (2). The TOU growth curves generated by these procedures, for each individual microbe, are reported as Images 1 A-E. This work was performed by Rhyz Labs, Provo, UT. (3). The challenge microbe counts from Rhyz Labs listed as Reported Values and ISE calculated values are reported in (Table 1).

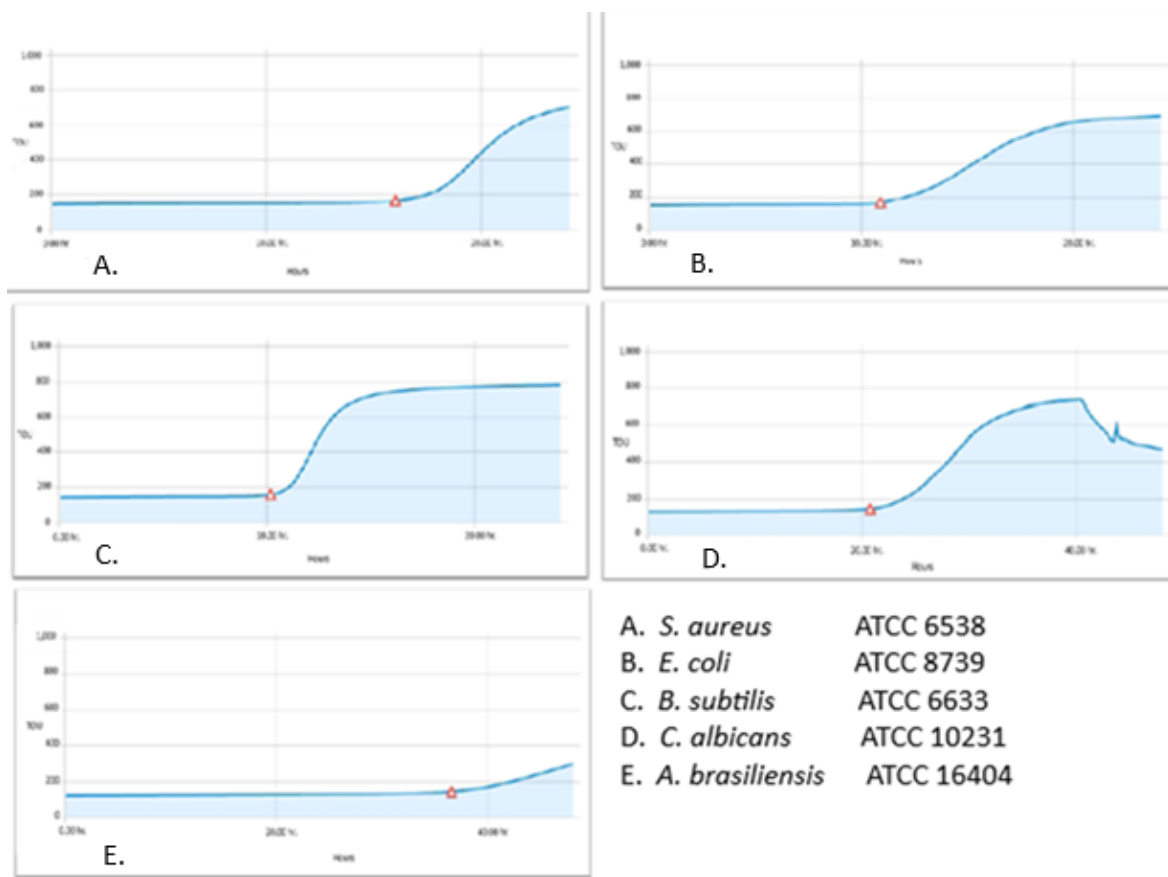Table 1 Challenge microbes with Reported and ISE calculated concentrations

Name		Reported Values	ISE Calculated Values
S. aureus	ATCC 6538	~1 cfu/mL	2 cfu/mL
E. coli	ATCC 8739	~2 cfu/mL	3 cfu/mL
B. subtilis	ATCC 6633	~1 cfu/mL	2 cfu/mL
C. albicans	ATCC 10231	~3 cfu/mL	2 cfu/mL
A. brasiliensis	ATCC 16404	~3 cfu/mL	1 cfu/mL

Five different species of microorganisms were grown and diluted to a level of 1-3 cfu/mL. 1mL from each solution of the low-level microorganisms was spiked into Soleris TVC- SA, EC, BS and YM-109-CA, BA rapid detection vials and tested at a specification of <1 cfu/mL.

The System read the light transmittance passing through the vials' sensors and generated the reported growth curves based on the TOU values created during each microorganism's assay. A sterile, blank vial typically starts with a baseline of approximately 130-150 TOU, ranging from 100-200 TOU. As the assays continued, TOU values increased as viable microbes were present and producing CO_2. The plotted TOU values vs time created a sigmoidal-like growth curve representative of the stationary, exponential growth, and lagging phases seen in typical microbial growth curves, Image 1.

TOU values were recorded at specified hours and used by the ISE method to generate the original microbial cfu/g of the sample. The triangle, found in all presumptive fail graphs, indicates the growth failure starting point or when the TOU's positive rate of change exceeds the software's failure parameters. It is not used in the ISE method.
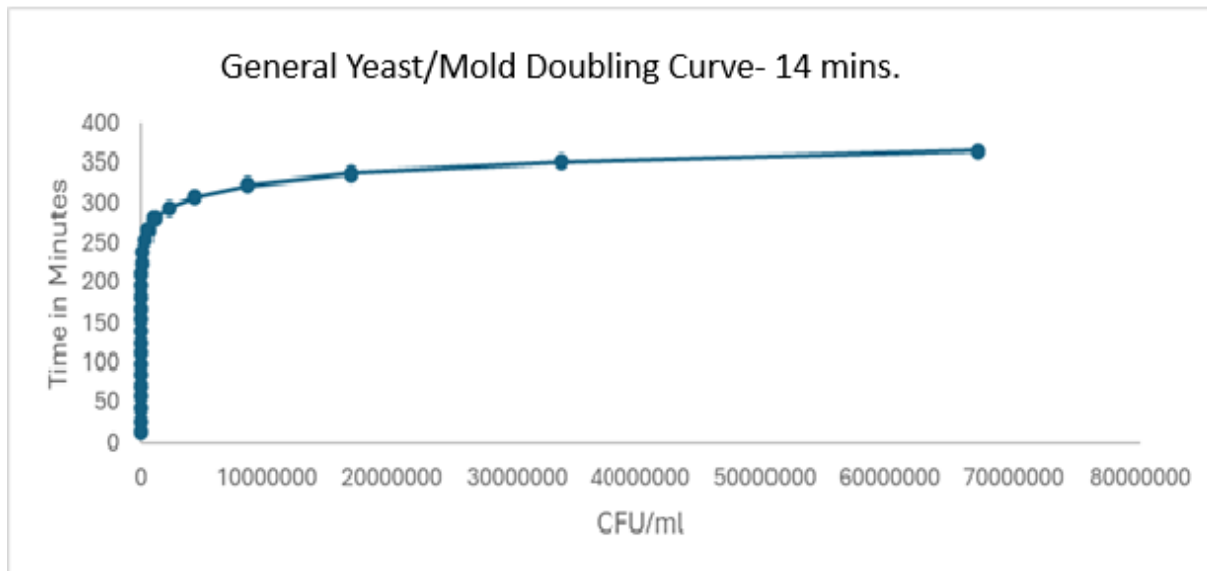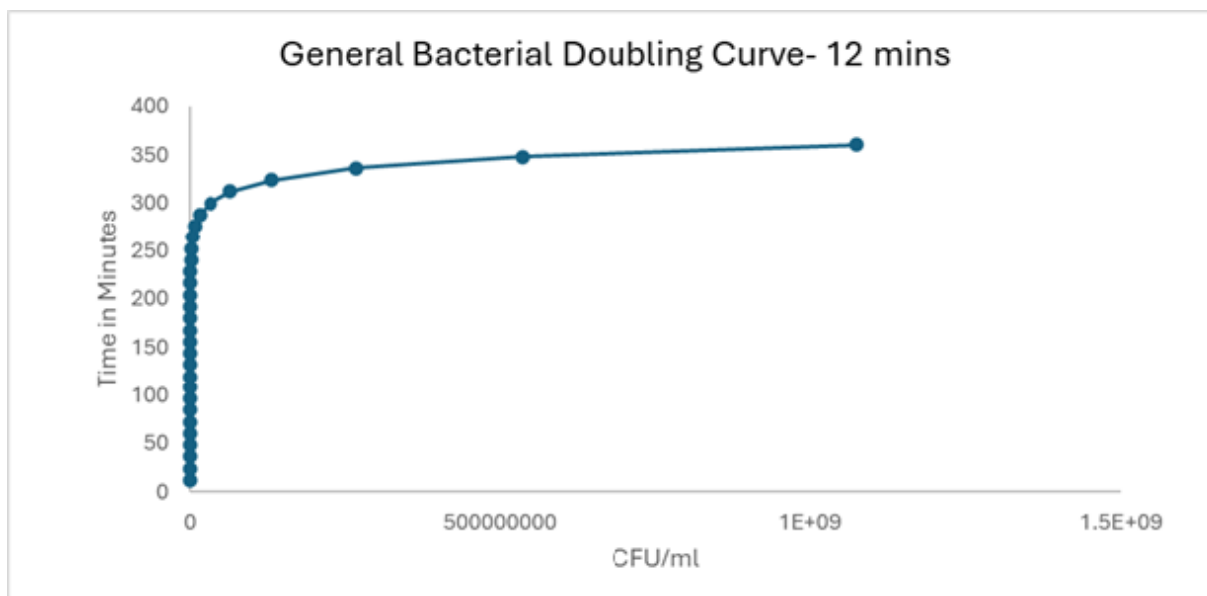
Image 1. TOU vs TIME Growth Curves of the Challenge Microbes

A.

B.

C.

D.

A. *S. aureus*   ATCC 6538
B. *E. coli*   ATCC 8739
C. *B. subtilis*   ATCC 6633
D. *C. albicans*   ATCC 10231
E. *A. brasiliensis*   ATCC 16404

E.

The System's growth curve is reported on a graph with TOU values plotted on the vertical axis and time (hours) on the horizontal. The Total Viable Counts (TVC) graph is divided into two ten-hour segments and a third, assay completed segment-not labeled. The TOU for Total Viable Counts is read from the graph at 0 hours, 10 hours, 20 hours and at the end of the assay. The same reading procedure applies for Yeast/Mold samples, except the graph is divided into two 20-hour periods and an assay-completed segment.   Yeast/Mold TOU values are read and recorded at 0 hours, 20 hours, 40 hours, and at the end of the assay. These four TOU values are then converted into cfu/g counts using the ISE method.

To link the TOU values to cfu/g the TOU/CFU ratio was created. To accomplish this, it was necessary to determine general growth doubling times for bacteria and yeast/mold. This was achieved by starting with one bacterial cell and allowing it to theoretically double every twelve minutes and one yeast/mold cell to theoretically double for fourteen minutes, Figure 1. The doubling times of twelve and fourteen minutes were chosen since they closely aligned with published cfu/mL counts (1). These doubling time values were used to calculate the TOU/CFU ratio.

Figure 1- Growth Doubling Curves

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General Bacterial Doubling Curve- 12 mins

Time in Minutes (y-axis, 0 to 400)
CFU/ml (x-axis, 0 to 1.5E+09)

General Yeast/Mold Doubling Curve- 14 mins.

Time in Minutes (y-axis, 0 to 400)
CFU/ml (x-axis, 0 to 80000000)

The TOU/CFU ratio is the foundation of the ISE method algorithm. For bacteria and yeast/mold it was calculated to be 0.0293, meaning that 1 cfu of bacteria is approximately equivalent to 0.0293 TOU and 1 cfu of Yeast/Mold is approximately equivalent to 0.0391 TOU. An initial TOU/CFU ratio was calculated at 0.002; however, this value, based on a doubling time of twenty minutes, was later proven to be incorrect since growth values were not supported by published results and subsequent plate confirmation testing. The TOU/CFU ratio needs a defined time threshold otherwise the number is arbitrary and undefined. The TOU/CFU ratio was calculated using the number of cfu generated at two hours using the doubling times previously mentioned. These reported TOU/CFU ratios were confirmed by third-party testing (Table 1) and aligned with the established CO_2 concentrations and CO_2 OD600 values previously published (1).

Useful Variables and Equations:

Bacteria $0.01mg\ CO_2 \approx 100,000\ cfu/mL$
            $0.01mg\ CO_2 = 0.001\ OD\ 600\ units$
            $0.01mg\ CO_2 = 2930\ TOU$
            $1\ cfu/ml \approx 0.0293\ TOU$

Yeast/Mold $0.01mg\ CO_2 \approx 10,000\ cfu/mL$
            $0.01mg\ CO_2 = 0.001\ OD\ 600\ units$
            $0.01mg\ CO_2 = 391\ TOU$
            $1\ cfu/mL \approx 0.0391\ TOU$

How ISE works- the test vial contains 1mL from the required test dilution and becomes the 'test plate' for the ISE method. All TOU readings come from the test vial. The ISE algorithm reads the plotted TOU values generated by the System with the Soleris Fusion Software and then uses those values to calculate the original cfu/g of the sample.

Not all samples have the same testing specifications. To account for these different test specifications the ISE Method uses the dilution scheme outlined in Table 2. This table lists the required Dilution Coefficients needed to normalize the TOU growth curve at the required test dilution. Without the correct Dilution Coefficient, the TOU values fall outside the reported graph parameters, adversely affect the internal standards, and prevent the ISE Method from reporting cfu/vial counts. The cfu/vial counts should mimic the cfu/plate counts derived from Direct Method Enumeration procedures at any given dilution. The Dilution Coefficient makes this possible.

The Dilution Coefficient values are based on a 10-gram test sample diluted into 90mL of diluent with 1mL being used in the test vial. This procedure creates a 1:10 dilution of the original sample and becomes the working sample for all subsequent dilutions. The Dilution Coefficient of 0.1 is used for this sample preparation since 1/10th of the original cfu/g count is found in the test vial. A Dilution Coefficient of 0.01 is used for a 1:100 dilution since 1mL from this dilution contains 1/100th the cfu/g of the original sample and so forth with each subsequent dilution. These Dilution Coefficient values become useful when verifying the cfu/vial (plate) at the given test dilution (specification). A Dilution Coefficient of 1 is used when the sample is tested as is and not diluted, (i.e. water), and also for any sample that does not dissolve when added to diluent (i.e. environmental swabs).

Table 2. Reported Dilution Coefficient Values for the required test dilutions using the ISE method.

Dilution Coefficient	Dilution	Test Condition
1	1	Non-diluted samples tested as is- water
1	10	Non-dissolved samples- environmental swabs
0.1	1:10	Diluted samples (Dissolved sample)
0.01	1:100	Diluted samples (Dissolved sample)
0.001	1:1,000	Diluted samples (Dissolved sample)
0.0001	1:10,000	Diluted samples (Dissolved sample)
0.00001	1:100,000	Diluted samples (Dissolved sample)

The Dilution Coefficient Values are based on a beginning sample of 10g diluted to a 1:10 concentration in 90mL of diluent and that 1mL from this diluted sample, and from subsequent dilutions are being analyzed in the test vial. 1mL from tested 'as is' samples will be placed directly into the test vial.

Results: The ISE method was applied to the five challenge microorganisms previously reported (Table 1). This analysis fine-tuned the TOU/CFU ratio, since the starting cfu/mL values were already known. The value of 0.0293 was used for the Total Aerobic Count and the value of 0.0391 was used for the YM calculations. Once the TOU/CFU values were confirmed, several samples with unknown starting cfu/g concentrations were tested using the ISE method.

These unknown samples were run on the System located in the Biomedical Research Microbiology Laboratory in Sandy, Utah. Soon after the System reported presumptive fails for each product, plate confirmation counts were performed by an independent, third-party, testing laboratory following USP prescribed, total aerobic count plating procedures (4) or performed in-house following Biomedical Research's prescribed total aerobic plate count procedures (5).

The samples' original cfu/g results from these two labs' plate procedures are listed as Plate Reported cfu/g. The ISE calculated cfu/g values of those same samples are listed as ISE Calculated cfu/g. The cfu/g were converted to Log values for both data sets and the Log Difference between the two Methods' counts was compared and reported in Table 3.

Table 3. Plate results compared to ISE calculated results

Sample	Plate Reported cfu/g	Log Values	ISE Calculated cfu/g	Log Values	Log Difference
1	30	1.48	10	1.00	-0.48
2	20	1.30	10	1.00	-0.30
3	1500	3.18	1660	3.10	0.04
4	600	2.78	1584	3.16	0.42
5	3400	3.53	1333	3.12	-0.41
6	70	1.85	82	1.91	0.07
7	130	2.11	126	2.10	-0.01
8	90	1.95	101	2.00	0.05
9	190	2.28	136	2.13	-0.15
10	40	1.60	90	1.95	0.35
11	170	2.23	98	1.99	-0.24
12	3000	3.48	1847	3.27	-0.21
13	100	2.00	147	2.17	0.17
14	<1000	3.00	1476	2.17	0.17
15	1000	3.00	1073	3.03	0.03
16	<100	2.00	23	2.00	0.00
17	<100	2.00	20	2.00	0.00
18	<1000	3.00	223	3.35	0.35
19	28500	4.45	16307	4.21	-0.24
20	<1000	3.00	804	3.00	0.00
21	<1000	3.00	179	3.00	0.00

The ISE calculated cfu/g results were compared to the plate reported cfu/g with a 95% Confidence LCL 1.36, UCL 3.71 for Reported Log values and 95% Confidence LCL 1.35 and UCL 3.69 for ISE Method Log values. The Log values for any of the ISE Calculated samples that had plate results of <100, or <1,000 were unchanged from the plate's log values to maintain consistency and allow for statistical analysis.

To compare method-result correlations, log conversions of reported cfu/g results are necessary since cfu are estimates of microbial growth. The accepted practice to accurately compare counts across different enumeration methods is via log values. The reported cfu/g are converted to log values, and the difference of those log values is then calculated. A ±0.5 log difference between different test method results is considered statistically similar. The ISE calculated values were all within a ± 0.5 log difference of the Reported cfu/g values.

When the Reported Log values were plotted against the ISE calculated Log values (Fig. 2), the R^2 result was 0.9146 demonstrating an acceptable R^2 value >0.9025. These results indicate that the ISE method can achieve counts similar to plate methods and points to the accuracy of the method. Also, these results raised some interesting questions.

Fig. 2 Plate Log Results vs ISE Calculated Log Results
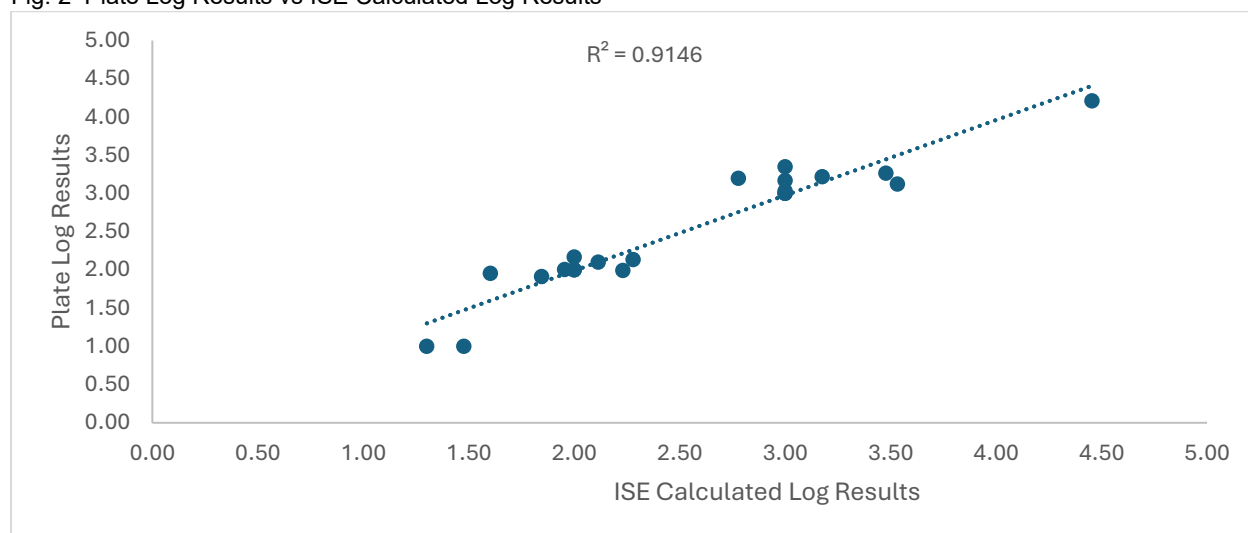
Could the ISE method predict counts in samples? Could it be used to explain plate results and develop test specifications? To answer these questions, the plate counts from three different lots of the same product with failing plate counts were compared. This work was performed by an off-site, third-party lab. Each sample was plated with a test specification of <10,000 cfu/ml. Theoretical TOU curves were generated to explain the observed plate results and reported as Theoretical Results. A day after the theoretical curves were generated the same three samples were analyzed on the System and enumerated using the ISE method to produce the Actual Results. The Theoretical results and TOU values were compared to the third-party's reported plate counts, and the Actual Results with their respective TOU values. The results of this experiment are reported in Table 4. Theoretical TOU values were picked that would generate the exact reported plate counts. Only the 1:1,000 dilution counts were used to quantify the final cfu/g count since all the reported 1:10,000 dilution counts were 1 cfu. The Log Difference values between the Theoretical and Actual log values for each sample were all well below the ±0.5 log requirement.

Table 4. Theoretical ISE Method vs. Actual ISE Method

THEORETICAL ISE METHOD

Sample ID	Plate count (cfu/g)	Plate count Log	TOU 0hr.	TOU 10 hr.	TOU 20 hr.	TOU End	Dilution 1:1000	Dilution 1:10,000	Plate count (cfu/g)	Log Values
AA15188	13,000	4.11	170	180	281	314	13	1	13,005	4.11
AA15189	15,000	4.18	160	170	330	409	15	1	15,002	4.18
AA15190	13,000	4.11	180	190	295	310	13	1	12,997	4.11

ACTUAL ISE METHOD

Sample ID	Plate count (cfu/g)	Plate count Log	TOU 0hr. Rep 1 Rep 2	TOU 10 hr. Rep 1 Rep 2	TOU 20 hr. Rep 1 Rep 2	TOU End Rep 1 Rep 2	Dilution 1:1000	Dilution 1:10,000	Plate count (cfu/g)	Log Values
AA15188	13,000	4.11	152/152	152/152	299/249	371/327	13	1	12,798	4.11
AA15189	15,000	4.18	155/155	155/155	275/242	362/339	13	1	12,569	4.1
AA15190	13,000	4.11	155/167	155/167	267/170	348/178	11	1	10,759	4.03

Log Difference values of ≤0.08 were calculated for the three samples. All values were calculated using an Excel spreadsheet without rounding. Actual ISE Method results are the average of two replicates. Sample AA15190 Rep 2 demonstrated no positive growth curve when tested via the Soleris System.

Since the ISE method accurately calculated final counts, could it accurately calculate the cfu/vial (plate) at various dilutions of the same sample? For the ISE Method, the test vial is used in place of an agar plate and the detected cfu's in the test vials should mimic the number of cfu's observed on plates from the same dilutions. This additional ISE method confirmation was achieved by performing Total Plate Counts at different dilutions of a single sample and comparing those counts to the ISE method generated counts at the same dilutions. The results of this experiment are reported in Table 5.

Table 5. Plate vs. ISE Method results- single sample assay at multiple dilutions.

Sample Identifier	Dilution	Observed cfu	Reported cfu/mL	Log Values	Average Log Values	Log. Diff
AA14358- Plate Method	0.01	TNTC	TNTC	NA	4.66	-0.38
	0.001	52	52,000	4.72		
	0.0001	4	40,000	4.60		
AA14358- ISE Method	0.01	183	18,309	4.26	4.28	
	0.001	20	19,629	4.29		
	0.0001	2	18,936	4.28		

TNTC- Too numerous to count. The plates were overgrown, and individual colonies could not be counted. The average cfu from three different plates are reported at each dilution for the Observed cfu and Reported cfu/mL values for the Plate Method.

The Observed cfu were converted to Reported cfu/mL by multiplying the Observed cfu by the required ISE Dilution Coefficient and the log values were then calculated and averaged.

The Log Difference between the two methods of -0.38 is within the ±0.5 acceptance range. Both data sets demonstrated similar log values and the counts from each subsequent dilution were a tenth of the previous dilution's count as expected. Most notably, even though subsequent 1:10 dilutions achieved diminishing cfu/mL results in the test vial, the final starting cfu/g of the sample remained relatively constant as expected. This experiment demonstrated that the ISE method could easily quantify microbial growth at various dilutions and points to the sensitivity of the method.

Next, the robustness of the ISE method was analyzed by assaying five random samples tested at dilutions ranging from 1:1,000 to 1:100,000 but still showing the same flatline, growth curves.

If the method works correctly across different product matrices and dilution levels, the same result should be generated for each sample with only the Dilution Coefficient and actual dilutions as variables. Within this test group three independent samples all shared the same <1,000 cfu/g specification. All five samples were analyzed with the ISE method at a 1:1,000 dilution level. Since each sample's graph generated no visible, positive rate of change, the TOU values for each sample should be identical and the test results should be ≤1 cfu/vial with the reported cfu/g sample count being ≤1000 cfu/g- this being the lowest, tested dilution. The results of this assay are reported in Table 6.

Table 6. ISE Method- Multiple Samples, Multiple Dilutions, Identical TOU Values

Sample ID	Dilution	TOU READS			cfu/vial	Sample cfu/g
		10 hrs.	20 hrs.	End		
AA12006	100,000	150	150	150	0	673
AA12010	10,000	150	150	150	0	673
AA12052	1,000	150	150	150	1	673
AA12056	1,000	150	150	150	1	673
AA12014	1,000	150	150	150	1	673

As predicted, counts were not observed in the samples' vials with dilutions >1,000 since the microorganisms were removed or diluted out of the assay. Since fractions of colonies are not possible and don't exist naturally, the ISE method rounds to the nearest whole number.

Even though counts are reported at the 1:1,000 dilutions this is due to the ISE method rounding up to the nearest whole number of 1 with a calculated cfu/vial result of 0.67. The reported value of 673 cfu/g is theoretical since it falls below the specified dilution of 1:1,000 with no positive rate of change in the growth curve. However, this number represents the highest possible cfu/g value, based on the reported TOU values. If growth was detected it would meet the <1,000 cfu/g specification with the reported result of 673 cfu/g. These results show that the ISE method is consistent across different product matrices and dilutions and briefly demonstrates the robustness of the ISE method.

Additional experiments were conducted to determine the precision of the method. The goal was to determine how much variation in TOU values could exist between graphs generating the same cfu/g results. Variation calculations were performed using a Dilution Coefficient of 0.1 and a 1:10 dilution. Ten different tests were performed using baseline TOU values ranging from 120-230 TOU increasing the TOU vertically by ten units each test, Table 7. This experiment determined that a vertical variance of 20 TOU is required to notice a change in sample cfu/g counts, otherwise any variation of <20 TOU reports the same sample cfu/g counts.

Table 7. TOU unit precision within the ISE Method

TOU Value Variation Calculation- 1:10 Dilution				
10 Hour	20 Hour	End	Result cfu/vial	Sample cfu/g
210	220	230	1	10
200	210	220	1	9
190	200	210	1	9

180	190	200	1	8
170	180	190	1	8
160	170	180	1	8
150	160	170	1	7
140	150	160	1	7
130	140	150	1	6
120	130	140	1	6

Reported cfu/vial and original sample cfu/g values using a 10 TOU vertical variance at each individual read point. Vertical variance is the amount of TOU units needed to see a change in the original cfu/g counts at a single reading point (i.e., 10 hours).

Additionally, a vertical change of 10 TOU at all three reading points, at a 1:10 dilution results in no change of the cfu/vial count and a change of 1 cfu/g in the original sample. This variance changes logarithmically as different dilutions are tested (Data not shown).

Discussion:

ISE Method Abilities- this technology is an accurate way to generate counts without plating, allowing for results in forty-eight hours for yeast/mold and twenty-four hours for aerobic bacterial counts. It has the ability to quickly resolve the System's presumptive failing Total Aerobic Plate Count and Yeast/Mold enumeration assays. This eliminates the need for retests, additional confirmation testing and costly plate preparation procedures. The ISE Method generates data in real-time, preliminary counts can be obtained during the assay; this allows the samples' count to be monitored during the assay.

ISE can generate counts ranging from 1 cfu/g to 4.5 million cfu/g in test samples for Total Aerobic and Yeast/Mold counts. The only information required from the analyst is the Dilution Coefficient, test dilution, and the TOU values from the completed assay graph. With this information the analyst can achieve Total Aerobic and Yeast/Mold starting cfu/g concentrations for most samples.

Test method suitability is generally not required for the ISE method since the measurement criteria generating the results, the vial's sensor, does not come into direct contact with the sample and is not adversely influenced by the test sample. The microorganisms either grow and produce CO_2 or they don't. However, care should be taken to identify any sample that may react with the vial's growth media leading to CO_2 production that may interfere with any present microbes' CO_2 generation leading to falsely exaggerated counts.

In theory, the ISE method could be used with any instrument that uses CO_2 for microbial enumeration after ascertaining the observable light transmittance to the CO_2 concentration for that device. It is currently optimized to work with Neogen's Soleris Microbiological Rapid Detection System with Fusion software.

Since the TOU curve is divided into three distinct growth phases, leading, exponential, and lagging, the ISE method uses three different TOU/CFU ratios to enumerate the microbes in each of these three growth phases. This dynamic TOU/CFU ratio approach is used to generate more accurate counts by fitting the TOU/CFU ratio to each unique aspect of the growth curve.

The starting TOU value is not used in calculating the final CFU/g result since this result is within the instrument's shut-eye stage and is only used as the initial baseline value. By using this starting TOU (0 hr.) time point value the ISE method can monitor cfu in real time as counts per vial over time or as a theoretical Internal Control highest probable final count per sample. This allows the user to create realistic bioburden specifications more quickly than using real time or accelerated aging studies.

Additionally, if the starting cfu/g count is less than the specified dilution the result can be reported as less than the specification or the actual count can be reported. The ISE method allows for real-time assessment of presumptive failures so that these failures can be resolved immediately upon completion of the assay.

It should be noted that several hours of time may all contain the same TOU value, thus CFU/TOU should not be expressed in the same way as CFU/hour, but a CFU count at any given hour is achievable if the TOU unit for that time point is known. The change in CFU is based on the change in TOU during a prescribed amount of time. For this reason, it is possible to achieve a cfu/g count at any given time point without a completed assay.

Any assays that appear to generate a quick rise in TOU during the assay's shut-eye stage and then levels off with no subsequent positive rate of change in the growth curve, should be considered as non-detectable growth and less than the assay specification.

Additional TOU/CFU ratios were linked together to allow for the enumeration of metabolically slower growing microorganisms or faster ones. The ISE method incorporates TOU/CFU ratios that mimic the growth of metabolically faster and slower microbes and averages these cfu counts to generate the final cfu/g sample count. Using multiple TOU/CFU ratios allows for the enumeration of slower growing microorganisms that may be overlooked and therefore not counted due to the presence of faster growing microorganisms that overgrow on traditional solid media plates. This adds a level of assurance to the final result. This also helps to overcome the shortcomings of counting less visible colonies growing on plates and allows the ISE method to enumerate growth that might otherwise not be visible during the required growth period.

ISE Method Limitations- This method is not suitable for assays that do not use CO_2 for growth measurements such as enzyme reaction detection or chemical synthesis reactions. It should not be used for enriched samples but may be used on non-enriched, unadulterated, pathogen samples if a count is required.

The TOU generated growth curve cannot be used for identification purposes. A particular microorganism genus/species cannot be identified simply by the shape of the growth curve, nor can the ISE method quantify or distinguish different bacterial genus within a heterogenous sample. It also can't quantify or distinguish individual species within a genus. However, the ISE method could be linked to identification technology potentially allowing a single instrument, in theory, to identify and enumerate microorganisms based on colony morphology or other identifying characteristics if the sample being tested is a pure culture.

Future work- To adapt the ISE method to specific genera of bacteria such as Enterobacter and coliforms that also require enumeration in some applications. This work also lays a solid foundation for the potential use of dilution absent microbiological enumeration techniques that we would like to further develop.

Closing Remarks- It is the author's belief the future of microbiology testing will demand microbial testing assays that combine the accuracy of direct method results with the speed of indirect testing methods. Additionally, if these newly developed methods use less resources than currently employed direct enumeration methods this adds additional benefit and reduces the testing's environmental footprint. This paper reports the capabilities, limitations and the future potential of the novel enumeration method, In-silico Enumeration- ISE.

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CONFLICT OF INTEREST:

The author declares no conflict of interest.

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