MICROBIOLOGICAL METHODS

Validation of the Soleris® NF-TVC Method for Determination of Total Viable Count in a Variety of Foods

*Performance Tested Method*SM 071203

Abstract

A study was conducted to determine the efficacy of the Soleris Non-fermenting-Total Viable Count (NF-TVC) automated growth-based method for semiquantitative detection of mesophilic, aerobic microorganisms in a variety of food products. A probability of detection (POD) statistical model was used to compare Soleris results at multiple test thresholds (dilutions) with aerobic plate counts determined using reference dilution plating procedures. Nine naturally contaminated food products were tested, with Soleris testing performed at three or four threshold levels for each food. Using the POD model, all Soleris test results were in statistical agreement with the reference plating procedures with the exception of a single threshold level in two trials with black pepper, and a single threshold level in the independent laboratory trial with cheesecake. Results of ruggedness testing showed that the Soleris method produced accurate results even when minor variances in operating parameters, including sample volume and incubation temperature, were introduced. Results of the internal and independent laboratory validation studies showed that the Soleris NF-TVC method can be used as an accurate alternative to conventional dilution plating procedures for evaluation of microbial counts at threshold levels, while saving 24 h or more in analysis time.

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The method was independently tested, evaluated, and certified by the AOAC Research Institute as a *Performance Tested Method*SM. *See* http://www.aoac.org/testkits/steps.html for information on certification.

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Scope of Method

Target organisms.—Aerobic, mesophilic bacteria, yeasts, and molds.

Matrixes.—The following foods were tested: raw chicken, deli ham, lettuce, almonds, black pepper, cheesecake, ice cream mix, nonfat dry milk, and cocoa powder.

Summary of validated performance claims.—Statistically equivalent to the AOAC (1) or *Standard Methods for the Examination of Dairy Products* (2) dilution plating aerobic count methods for evaluation of microbial counts at threshold levels using a probability of detection (POD) model.

Definitions

POD (obs.).—Observed POD for the test method; number of test method positive results divided by the number of portions tested.

POD (pred.).—POD for the test method predicted from the reference method result. POD (pred.) = $1 - e^{-c}$, where e is the base of the natural logarithm (value = 2.7183) and c is the number of input CFU/test based on the reference method result.

Pass/fail result.—If the POD (pred.) falls within the lower and upper 95% confidence limits of the POD (obs.), the result is pass; otherwise, it is fail.

Soleris test threshold.—The detection limit of the Soleris method determined by the volume and dilution of test sample homogenate added to the Soleris vial. For example, a threshold of >100 CFU/g represents 1 mL of a 1:100 dilution added to the vial, equivalent to 0.01 g of original test sample.

General Information

Standard methods for detection or enumeration of total aerobic bacteria, yeasts, and molds in foods are based on dilution plating, filtration, or most probable number approaches. Examples of dilution plating methods include AOAC *Official Method*SM **966.23** (1) and the standard plate count method from *Standard Methods for the Examination of Dairy Products* (2). These methods require 48 h to obtain results. The Soleris method, through sensitive determination of metabolic activity during microbial growth, produces results within 24 h for most foods.

Principle

Soleris Non-fermenting Total Viable Count (NF-TVC) is a growth-based, automated method with an optical endpoint. A dilution of the test sample homogenate is inoculated directly into the test vial. The prepared vial is then placed in the Soleris instrument set at the appropriate incubation temperature. As microorganisms grow and metabolize, carbon dioxide is produced that diffuses from the growth medium through a gas-permeable layer and into the indicator portion of the Soleris vial. Dissolved carbon dioxide leads to the formation of carbonic acid, reducing the pH and resulting in a change in color of the chemical indicator over time. The color is monitored by the instrument; a change in color of a certain magnitude, determined by the Soleris software, indicates a positive test result. The Soleris instrument contains temperature-controlled incubation chambers and photodiode-based optical detection devices for measurement of color changes in the bottom indicator portion of the vial.

The Soleris NF-TVC vial represents an improvement over the original Soleris TVC vial. The indicator system has been changed to utilize detection of carbon dioxide rather than detection of acid production, expanding the inclusivity of the vial to include non-fermenting organisms. The Soleris NF-TVC test can be used in a "dilute-to-specification" or threshold manner in which the result is positive or negative around a desired cutoff (in CFU/g) determined by the dilution and volume of sample homogenate added to the vial. It is assumed that 1 CFU introduced into the Soleris vial will lead to a positive result. Alternatively, the test can be used in a fully quantitative manner by building product-specific calibration curves relating initial analyte concentration to Soleris detection time. Use of the test with calibration curves is outside the scope of this validation study.

Test Kit Information

(**a**) *Test name*.—Soleris® NF-TVC, 9 mL.

(**b**) *Cat. No*.—NF-TVC.

(**c**) *Ordering information*.—*Inside the United States*.—Neogen Corp., 620 Lesher Pl., Lansing, MI 48912, Tel: 517-372-9200, Fax: 517-372-0108, Website: www.neogen.com. *Outside the United States*.—Contact above office for local distributor information.

(**d**) *Soleris NF-TVC vial*.—Sterile growth medium (9 mL) in a plastic vial, in boxes of 100 vials. One test sample/vial. Store at 2–8°C. Requires use of the Soleris instrument.

Supplies and Reagents

Additional supplies and reagents available from Neogen Corp.

(**a**) *Soleris 32 (Product No. BS32) or Soleris 128 (Product No. BS128) instrument*.—Containing one or four temperature-controlled (15–60 ± 0.5°C) incubator drawers, respectively, with 32 locations in each drawer. Each test location contains a light emitting diode (LED)/based optical sensor for measurement of changes in absorbance over time. Includes dedicated computer, monitor, and software.

(**b**) *Stomacher®-type bags*.—With filter (Product No. 6827).

(**c**) *Balance*.—For weighing samples, minimum 100 g capacity, ± 0.1 g.

(**d**) *Micropipettor and tips*.—100–1000 µL.

Standard Reference Materials

Not applicable.

Standard Solutions

Not applicable.

Safety Precautions

Use of this test should be restricted to individuals with appropriate laboratory training in microbiology. Reagents are for laboratory use only. All pipetting transfers should be made using either a disposable pipet and pipetting aid or micropipet with disposable tips. Refer to the Material Safety Data Sheet from Neogen Corp. for more information. Used Soleris vials, sample homogenates and dilutions, and pipets or pipet tips should be handled and disposed of as potentially infectious material. The preferred method for disposal of contaminated materials is autoclaving. Items that cannot be autoclaved may be decontaminated by treatment with a disinfectant solution, for example 10% bleach, followed by rinsing with water.

Soleris Method

Sample Preparation

(**a**) Prepare a 1:10 sample homogenate (e.g., 50 g + 450 mL diluent) using Butterfield's phosphate-buffered dilution water (PBW). Mix, shake, or homogenize the sample as appropriate.

(**b**) Prepare dilutions of the sample homogenate to achieve the desired test threshold, e.g., for >10 CFU/g, use sample homogenate as is. For >100 CFU/g, prepare further 1:10 dilution in PBW. For >1000 CFU/g, prepare further 1:100 dilution, etc.

(**c**) Add 1.0 mL sample homogenate or dilution to the Soleris vial. Cap the vial tightly and invert three times to mix. Back off the vial cap to allow air exchange.

(**d**) Proceed to Soleris analysis.

General Preparation

The test should be performed under normal laboratory conditions with respect to humidity, temperature, lighting, etc. Soleris vials should not be used beyond their expiration date.

Soleris Analysis

Note: The Soleris system requires installation and user training. Both are provided by Neogen Corp.

(**a**) *In the Soleris software menu*.—Select an incubator drawer. The incubation temperature should be set to 35 ± 0.5°C for nondairy products or 32 ± 0.5°C for dairy products.

(**b**) *On the sample position grid*.—Select the test (NF-TVC) and drag/drop to the selected sample position.

(**c**) *On the sample queue screen*.—Enter the following: sample identification number, and, if desired, production lot number, plant information, and user name.

Table 1. Comparative testing results and probability of detection calculations for the Soleris NF-TVC method

Food type	Reference plate count (CFU/g)[a]	Soleris test threshold (CFU/g)[b]	CFU/vial[c]	Predicted POD[d]	Soleris result		Observed POD[f]			Interpretation[g]
					No. of vials positive[e]	No. of vials tested	LCI	POD	UCI	
Raw chicken	8.7×10^6	1000000	8.7	0.999	20	20	0.832	1	1	Pass
		10000000	0.87	0.581	9	20	0.258	0.450	0.678	Pass
		100000000	0.087	0.083	1	20	0.009	0.050	0.236	Pass
Raw chicken[h]	6.5×10^3	100	65	1	20	20	0.832	1	1	Pass
		1000	6.5	0.998	20	20	0.832	1	1	Pass
		10000	0.65	0.478	10	20	0.299	0.500	0.701	Pass
Deli ham	1.3×10^3	100	13	1	20	20	0.832	1	1	Pass
		1000	1.3	0.727	12	20	0.387	0.600	0.781	Pass
		10000	0.13	0.122	0	20	0	0	0.168	Pass
Lettuce	1.8×10^4	1000	18	1	20	20	0.832	1	1	Pass
		10000	1.8	0.835	16	20	0.584	0.800	0.919	Pass
		100000	0.18	0.165	1	20	0.009	0.050	0.236	Pass
Almonds	2.0×10^2	10	20	1	20	20	0.832	1	1	Pass
		100	2.0	0.865	16	20	0.584	0.800	0.919	Pass
		1000	0.2	0.181	2	20	0.028	0.100	0.301	Pass
Black pepper	2.9×10^3	1000	2.9	0.945	20	20	0.832	1	1	Pass
		10000	0.29	0.252	9	20	0.258	0.450	0.658	Fail
		100000	0.029	0.029	0	20	0	0	0.168	Pass
Black pepper	1.3×10^3	100	13	1	20	20	0.832	1	1	Pass
		1000	1.3	0.727	9	20	0.258	0.450	0.658	Fail
		10000	0.13	0.122	4	20	0.081	0.200	0.416	Pass
		100000	0.013	0.013	1	20	0.009	0.050	0.236	Pass
Cheesecake	1.2×10^5	10000	12	1	20	20	0.832	1	1	Pass
		100000	1.2	0.699	15	20	0.531	0.750	0.888	Pass
		1000000	0.12	0.113	2	20	0.028	0.100	0.301	Pass
		10000000	0.012	0.012	0	20	0	0	0.168	Pass
Cheesecake[h]	1.1×10^7	1000000	11	1	20	20	0.832	1	1	Pass
		10000000	1.1	0.667	15	20	0.531	0.750	0.888	Pass
		100000000	0.11	0.104	14	20	0.481	0.700	0.854	Fail
Ice cream mix	3.6×10^4	1000	36	1	20	20	0.832	1	1	Pass
		10000	3.6	0.973	19	20	0.764	0.950	0.991	Pass
		100000	0.36	0.302	5	20	0.112	0.250	0.469	Pass
Nonfat dry milk	2.9×10^2	10	29	1	20	20	0.832	1	1	Pass
		100	2.9	0.945	19	20	0.764	0.950	0.991	Pass
		1000	0.29	0.252	2	20	0.028	0.100	0.301	Pass
		10000	0.029	0.029	1	20	0.009	0.050	0.236	Pass
Cocoa powder[i]	1.8×10^1	10	1.8	0.835	17	20	0.640	0.850	0.948	Pass
		100	0.18	0.165	6	20	0.145	0.300	0.519	Pass
		1000	0.018	0.018	1	20	0.009	0.050	0.236	Pass

[a] Direct plating methods: AOAC OMA **966.23** (nondairy products), *Standard Methods for the Examination of Dairy Products* method 6.020 (dairy products).

[b] Dilute-to-specification approach. Positive result expected if organism concentration in test dilution is greater than Soleris test threshold.

[c] CFU/Soleris vial based on reference plate count.

[d] Probability of detection (predicted): based on reference plate count ($POD = 1 - e^{-c}$).

[e] Detection times within 24 h verified by visual confirmation of indicator color change (blue-green to yellow-green or yellow) indicates a positive result at the test threshold selected.

[f] Probability of detection (observed): Fraction of Soleris results positive. LCI and UCI are 95% lower and upper confidence intervals, respectively.

[g] Test for equivalence of reference plate count and Soleris results. For "Pass," POD (pred.) must lie within POD (obs.) LCI–UCI range.

[h] Trial performed by independent laboratory.

[i] Soleris vials were incubated for 27 h.

(**d**) *Place the inoculated Soleris vials into the selected drawer locations*.

(**e**) *Once all the samples are entered*.—Select and click Start.

(**f**) *Test samples will incubate for 24 h*.—A detection curve will be generated in real time. The Soleris software will indicate a positive test result. Positive results will generally be indicated in less than 24 h.

Interpretation of Results

Negative criterion.—Tests producing no detection within 24 h are considered negative at the test threshold selected.

Positive criterion.—Detection times within 24 h verified by visual confirmation of indicator color change (blue-green to yellow-green or yellow) indicate a positive result at the test threshold selected.

Internal Validation Studies

Comparative Testing of Naturally Contaminated Foods

The Soleris NF-TVC method was compared to AOAC *Official Method of Analysis* **966.23** (aerobic plate count; 1) for the following foods: raw chicken, deli ham, lettuce, almonds, black pepper, cheesecake, and cocoa powder. The Soleris method was compared to method 6.020 of *Standard Methods for the Examination of Dairy Products* (standard plate count; 2) for ice cream mix and nonfat dry milk. All foods were naturally contaminated and were tested without inoculation.

Methodology

Sample preparation.—For each food type, a 1:10 sample homogenate was prepared in accordance with the appropriate reference method. Further dilutions were prepared for Soleris testing at three or more test thresholds and for the reference method plate count. Soleris test thresholds were chosen based on an estimate of the microbial load of the test sample and with the goal of achieving fractional positive results for at least one level, i.e., a target of 5–15 positive results out of 20 replicates assay at a particular dilution. This ensures that the Soleris method has been challenged at an inoculation level of approximately 1 CFU/vial or less.

Soleris testing.—For the Soleris method, 20 vials were inoculated with 1 mL dilution for each test threshold. Duration of the Soleris test was 24 h for all foods except cocoa powder, for which incubation was extended to 27 h.

Reference method plate counts.—Reference method aerobic plate counts were performed following AOAC *Official Method of Analysis* **966.23** (1) or *Standard Methods for the Examination of Dairy Products* Method **6.020** (2), except that the number of replicate plates at each dilution was increased to 10 to achieve a more accurate count. Standard counting rules were followed, i.e., counts were based on plates containing 30–300 colonies whenever possible.

Data analysis.—Results for each food type were analyzed using the POD model. The predicted POD for the Soleris method was calculated using the formula POD (pred.) = $1 - e^{-c}$, where c = CFU/vial at a given test threshold based on the reference method plate count result. The observed POD for the Soleris method was calculated at each test threshold by dividing the number of positive Soleris results by the number of portions tested (i.e., 20). Confidence intervals at a level of 95% around POD (obs.) were calculated using a POD interval calculator (P. Wehling, personal communication). If POD (pred.) was within the confidence interval of POD (obs.), then the Soleris and reference method results were considered not to be statistically different.

Example

Reference method result: 63 CFU/g

Soleris test threshold: >100 CFU/g

Soleris POD (pred.): $1 - e^{-0.63} = 0.467$

Soleris POD (obs.): 9/20 = 0.450

Soleris POD (obs.) with confidence intervals: 0.258–0.658

Result: Pass

Results

Results are reported in Table 1. Reference method aerobic plate counts ranged from a low of 1.8×10^1 CFU/g for cocoa powder to a high of 8.7×10^6 CFU/g for raw chicken. All trials produced fractional positive results at one or more test threshold levels with the Soleris method. Based on the POD analysis, there were no statistically significant differences in results between the Soleris and reference methods for eight of the nine foods at any test threshold.

In the initial trial with black pepper, POD (pred.) was slightly outside of the POD (obs.) lower confidence limit at the >10 000 CFU/g test threshold (0.252 versus 0.258). Results at both the >1000 and >100 000 CFU/g test thresholds passed the statistical test. A second trial with black pepper was conducted using four test threshold levels. In this trial, three of four levels passed the statistical test, with the intermediate level of >1000 CFU/g failing. At this level, POD (pred.) was marginally greater than the POD (obs.) upper confidence limit (0.727 versus 0.658). There is no obvious explanation for these discrepant results other than a statistical anomaly. Results argue against alternative hypotheses, such as matrix inhibition of microbial growth, as the cause. In the first trial, all 20 vials were positive at the >1000 CFU/g test threshold with a low input of 2.9 CFU/vial. Further, the discrepancy at the >10 000 CFU/g test threshold was in the direction of overestimation of the microbial population by the Soleris method.

For cocoa powder, initial experiments indicated that Soleris method sensitivity was inadequate with a 24 h test duration; therefore, a test duration of 27 h was used with good results.

Independent Laboratory Study

Methodology.—The independent laboratory performed two trials, one with raw chicken and one with cheesecake. Test protocols and data analysis methods were identical to those used for the internal validation studies.

Results.—Results are shown in Table 1. The aerobic plate count of the raw chicken sample was 6.5×10^3 CFU/g. Fractional positive Soleris results were obtained at one level, and POD (pred.) was within the confidence interval of POD (obs.) for all three test thresholds. The aerobic plate count of the cheesecake sample was 1.1×10^7 CFU/g. The data passed the statistical test at both the >1 000 000 and >10 000 000 CFU/g test thresholds (inputs of 11 and 1.1 CFU/vial based on the reference method plate count), but failed at the >100 000 000 CFU/g test threshold (0.11 CFU/vial), with POD (pred.) of 0.104 versus POD (obs.) confidence interval of 0.481–0.854. The reference method plate count was based on the 10^{-6} dilution (>1 000 000 Soleris test threshold) based on standard counting rules (mean count of 11 CFU from 10 plates). However, the 10^{-7} and 10^{-8} dilutions were also plated, producing mean counts of 2 and 1 CFU, respectively, suggesting an abnormality in the dilution series. If the 10^{-8} dilution contained on average 1 CFU/mL, then the Soleris result of 14 positives out of 20 replicates is within statistical expectations. Overall, results of the independent laboratory trials are consistent with those of the internal studies.

Ruggedness, Stability, and Lot-to-Lot Consistency Testing

Results of method ruggedness testing showed that changes in sample volume of ±20% and changes in incubation temperature of ±2°C had no significant effect on Soleris results. Detailed results are contained in the *Performance Tested Method*℠ (PTM) study report.

Real-time stability testing of three lots of Soleris NF-TVC vials established initial expiration dating of 6 months from date of manufacture. Details are available in the PTM study report.

Discussion

Results of these studies show that the Soleris NF-TVC method can be used as an acceptable alternative to reference dilution plating procedures for semiquantitative determination of mesophilic aerobic counts at designated threshold levels in a wide variety of food products. With few exceptions, Soleris method results were statistically equivalent to those of the reference plating procedures as determined using a POD model. Use of the Soleris NF-TVC method in a "dilute-to-specification" mode allows users to match test thresholds with product release specifications. Compared to dilution plating procedures, the Soleris method offers the advantages of minimal labor and reduced analysis time; results are obtained in 24 h or less compared to the 48 h required by the conventional plating procedures.

Conclusions

It is recommended that the Soleris NF-TVC method be granted PTM status for estimation of mesophilic aerobic bacteria, yeasts, and molds at designated threshold levels in raw chicken, deli ham, lettuce, almonds, black pepper, cheesecake, ice cream mix, nonfat dry milk, and cocoa powder.

References

(1) AOAC INTERNATIONAL (2005) *Official Methods of Analysis* online, AOAC INTERNATIONAL, Gaithersburg, MD, Method **966.23**, www.eoma.aoac.org

(2) American Public Health Association (2004) *Standard Methods for the Examination of Dairy Products*, 17th Ed., APHA, Washington, DC, Method **6.020**