CERTIFICATION

AOAC® *Performance Tested*SM

Certificate No.

031501

The AOAC Research Institute hereby certifies that the performance of the test kit known as:

MicroSnap Total

manufactured by

Hygiena LLC.
941 Avenida Acaso
Camrillo, California
USA

This method has been evaluated in the AOAC® *Performance Tested Methods*SM Program, and found to perform as stated by the manufacturer contingent to the comments contained in the manuscript. This certificate means that an AOAC® Certification Mark License Agreement has been executed which authorizes the manufacturer to display the AOAC *Performance Tested*SM certification mark along with the statement - "THIS METHOD'S PERFORMANCE WAS REVIEWED BY AOAC RESEARCH INSTITUTE AND WAS FOUND TO PERFORM TO THE MANUFACTURER'S SPECIFICATIONS" - on the above mentioned method for a period of one calendar year from the date of this certificate (December 18, 2018 – December 31, 2019). Renewal may be granted at the end of one year under the rules stated in the licensing agreement.

Scott Coates

Scott Coates, Senior Director
Signature for AOAC Research Institute

December 19, 2018
Date

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Hygiena, LLC.
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KIT NAME(S)
MicroSnap Total

CATALOG NUMBERS
Enrichment Device – MS1-TOTAL
Detection Device – MS2-TOTAL

INDEPENDENT LABORATORY
Silliker Laboratories
Chicago, Il
USA

AOAC EXPERTS AND PEER REVIEWERS
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APPLICABILITY OF METHOD
Target organism – Aerobic heterotrophic bacteria

Matrices – (50 g) - Fresh ground beef (<20% fat), raw chicken, pre-packaged iceberg leaves, fresh cream cake (17% fat), and raw cow's milk

Performance claims - The method was shown to have good correlation with International Organization for Standardization (ISO) method, ISO 4833:2003, *Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of microorganisms -- Colony-count technique at 30°C* (ISO 4833) (3) reference method for enumeration of TVC in the claimed matrices.

REFERENCE METHOD
ISO 4833:2003, Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of microorganisms – Colony-count technique at 30°C (3)

ORIGINAL CERTIFICATION DATE
March 25, 2015

CERTIFICATION RENEWAL RECORD
Renewed annually through December 2019

METHOD MODIFICATION RECORD
1. **December 2017 Level 1**

SUMMARY OF MODIFICATION
1. **Editorial changes**

Under this AOAC® *Performance Tested*℠ License Number, 031501 this method is distributed by:
NONE

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NONE

PRINCIPLE OF THE METHOD (1)

The MicroSnap Total system depends on the growth of bacteria in a proprietary enrichment media and the concomitant intracellular production of the diagnostic biomarker ATP. The mean intracellular ATP concentration at specific growth time points is then related directly to the starting inoculum of bacteria in the food matrix under measure. The assay uses a liquid stable based system to determine the level of bacterial ATP in the assay. The growth medium contains specific and rapid acting ATPase enzymes that modify and reduce the background ATP in the matrix. At specific time points, the concentration of intracellular ATP is directly proportional to the starting concentration of bacteria. The quantification point for TVC enumeration in this test is 7 hours at 30 ± 0.5°C. This time point allows enough dynamic range to support quantification over a range of starting concentrations.

The media used in the assay supports the rapid growth of all major foodborne bacterial populations and is buffered enough to support the final bioluminescence assay (all inhibitors of beetle luciferase bioluminescence have been removed). The media also contains two thermostable ATPase enzymes, which in the liquid media remain active and stable for 18 months when stored at 4°C. The ATPase enzymes remain active during the course of the incubation period, which in this case is 7 hours at 30°C. During this period, the enzymes act to degrade the ATP found extracellularly in the media from the food matrix. The 7 hour depletion phase is extremely efficient at removing the background to levels approaching low signal to noise ratios or true blank RLUs. This has been demonstrated in internal studies on a broad range of food types. Some example food types are listed in Table 1. These foods were considered problematic due to particulates, high protein and turbidity affecting the signal and the enzyme action. Not all food types reach the same RLU, but they all reach an equilibrium RLU above which the bacteria need to grow to be measured successfully in the assay.

The bacterial lysis agent used at the 7 hour assay time point is rapid enough to extract all bacteria, while inactivating the ATPase enzymes, allowing the intracellular bacterial ATP to be measured in a stoichiometric manner allowing a level of quantification to be done. In all raw and cooked foods tested internally, there was no increase in extractable ATP levels in the absence of a growing bacteria or eukaryotic organisms. Without a rate of change of active growth the system will not measure ATP increases.

At specific time points through the incubation, the concentration of intracellular ATP from the growing bacteria can be demonstrated. By adding bacteria to a selection of the foods, the depletion and subsequent growth of the inoculated bacteria can be tracked by measuring the ATP content using bioluminescence. The lytic component of the bioluminescence reagent efficiently inactivates the ATPase at the time of measurement with any active ATPase not deterring from the bioluminescence signal recovered. Figure 1 demonstrates ATP measurements in 10% milk, produced with and without ATPase, inoculated with a low number of *E. coli* at approximately 1000 CFU/mL. The ATP content was then estimated hourly by removing an aliquot for measurement. Using ATPases, the background signal is depleted before the bacteria grow sufficiently to be measurable above the depleted background. This is seen as a decrease in the RLUs from time 0 in the presence of ATPase's, and then an increase in the signal as the intracellular bacterial ATP becomes the dominant signal above the depleted extracellular ATP level.

PRINCIPLE OF METHOD CONTINUED (1)

The conversion of RLU to CFU was determined from a mixture of experimental data. Initial experiments were run using type cultures. By tracking the bioluminescent growth and comparing to the starting inoculum levels from plate counts, the conversions can be derived at each time point. Additionally, empirical data was used from naturally contaminated foodstuffs, including meat, vegetable and dairy products, again tracking the growth bioluminescently and comparing to starting inoculum as derived from plate counts. These experiments gave a good indication of how well the system will perform across many foodstuffs. The empirical data demonstrated that there can be a wide diversity of species from 24 to 72 h plate counts, and that those bacteria will grow equally well in the TVC-Broth and be available for extraction at each time point. Lastly, stressed or equilibrated bacteria were introduced onto sterile foods, such as baked products and aseptic infant formula, and again run in similar experiments tracking the bioluminescent growth and comparing this to the starting inoculum.

These experiments amalgamated into a correlation of best fit. A conversion can be based on where more than 90% of the correlation holds true to with a margin of ± 10%. The conversion at 7 hours is a simple multiplier of x10 (range x9 to x11) which brings the comparison to 1 = 1 for RLU/CFU to CFU from a standard method pour plates. At incubation time points other than 7 h, a conversion is also available from Hygiena.com via a simple Microsoft Excel® calculator, which takes into account the different incubation times.

DISCUSSION OF THE VALIDATION STUDY (1)

The MicroSnap Total detection system is primarily designed to give a rapid quantitative assessment for total viable counts of aerobic heterotrophic bacteria in food samples. The product is sold as a system for quantitation of medium to high levels of aerobic bacteria using a single 7 h incubation at 30 ± 0.5°C. By altering or extending the incubation time, the system can be adapted to estimate lower levels or higher levels of bacteria, but for standard levels of 100 to 50,000 CFU/g, the recommended incubation time is 7 h at 30 ± 0.5°C. The MicroSnap Total is the second of a pipelineof bioluminogenic assays from Hygiena designed to give the investigator the ability to determine levels of contaminating organisms in less than 8 hours. The unique nature of the assay producing light in relation to a biomarker concentration means the system has the ability to detect low levels of bacteria that are actively growing in the sample. If the biomarker follows a dose dependent response based upon the growth of the organism, then under standard conditions, the tracking of this biomarker at specific time points along the growth curve can give a good estimate of the starting inoculum.

The experimental work carried out in our laboratory and those of customers has supported the outcome that individual species of bacteria have a lesser impact than running empirical data on real foods and samples. The generation of ATP by individual species or genera does differ but not to the extent that it makes a real difference to total growth from contaminated food stuffs in comparative studies.

The populations seen on standard plate count and pour plate agars differ tremendously, depending on the source of the raw materials. However, major differences in growth can occur when comparing recoveries from very different samples or when the stresses on the bacteria are much greater, for example comparing equal inoculations onto dry surfaces compared to food stuffs, or from samples which have biocides added. This is when major growth differences and rates of production of intracellular ATP are seen. The response to stress is to extend the lag phase of all bacteria as opposed to differences between species at the same level of stress or inoculum level.

The levels of quantification required by users could be a traffic light type system for some investigators or threshold levels can be derived. The setting of these levels will be determined for each food by each investigator, but once installed the assay will reliably determine variations in the bacterial count.

Table 2. Matrix Study for the MicroSnap Total and ISO 4833 (1)

Matrix	Contam. level[a]	MicroSnap Total			ISO 4833			p-value[e]	Mean Diff.[f]	95% CI[g]		r^{2j}
		Mean[b]	S_r[c]	RSD_r[d]	Mean	S_r	RSD_r			LCL[h]	UCL[i]	
Lettuce	1	3.64	0.31	8.40	3.31	0.11	3.44	0.11	0.33	-0.31	0.41	0.948
	2	4.58	0.31	6.71	4.43	0.14	3.23	0.14	0.15	-0.26	0.28	
	3	4.77	0.16	3.32	4.75	0.17	3.50	0.81	-0.02	-0.27	0.27	
	4	5.75	0.18	3.17	5.58	0.07	1.18	0.22	0.17	-0.31	0.35	
	5	6.40	0.34	5.31	6.69	0.18	2.73	0.16	-0.29	-0.52	0.42	
Raw milk	1	3.18	0.16	5.12	3.05	0.03	0.87	0.51	0.13	-0.37	0.31	0.990
	2	3.35	0.04	1.16	3.53	0.19	5.32	0.33	-0.18	-0.47	0.43	
	3	5.02	0.02	0.41	5.07	0.08	1.64	0.39	-0.05	-0.15	0.14	
	4	6.16	0.50	8.17	6.15	0.04	0.72	0.95	0.01	-0.42	0.43	
	5	6.69	0.24	3.62	6.47	0.07	1.09	0.26	0.22	-0.41	0.48	
Raw chicken	1	3.40	0.11	3.17	3.20	0.02	0.60	0.19	0.20	-0.26	0.28	0.969
	2	3.90	0.03	0.79	3.92	0.01	0.18	0.79	-0.01	-0.34	0.34	
	3	4.46	0.40	8.98	4.18	0.13	3.11	0.13	0.29	-0.40	0.48	
	4	4.49	0.09	1.90	4.79	0.01	0.12	0.03	-0.30	-0.17	-0.14	

	5	5.16	0.16	3.11	5.08	0.05	0.90	0.29	0.08	-0.16	0.17	
	6	6.22	0.16	2.57	6.31	0.02	0.26	0.22	-0.09	-0.18	0.17	
	7	6.69	0.46	6.89	6.89	0.01	0.19	0.39	-0.20	-0.33	0.34	
Cream cake	1	2.64	0.21	7.95	2.62	0.31	11.80	0.21	0.02	-0.12	0.14	0.987
	2	3.31	0.07	2.03	3.45	0.03	0.93	0.11	-0.14	-0.11	0.10	
	3	4.69	0.05	0.97	4.69	0.01	0.05	0.98	0.00	-0.11	0.11	
	4	5.41	0.31	5.73	5.57	0.17	3.01	0.13	-0.16	-0.39	0.08	
	5	5.65	0.24	4.18	5.47	0.13	2.29	0.05	0.18	-0.15	0.17	
	6	6.27	0.85	1.56	6.61	0.03	0.46	0.39	-0.35	-0.44	0.35	
	7	6.28	0.29	4.63	6.51	0.03	0.40	0.23	-0.23	-0.49	0.41	
Raw ground beef	1	2.67	0.21	7.88	2.41	0.16	6.60	0.09	0.26	-0.45	0.47	0.7712
	2	3.89	0.17	4.07	3.59	0.31	8.54	0.18	0.36	-0.41	0.48	
	3[k]	4.26	0.32	7.45	3.72	0.00	0.02	0.25	0.53	-0.85	0.91	
	4[k]	4.37	0.36	8.22	2.86	0.00	0.03	0.11	1.52	-0.71	1.08	
	5	4.87	0.68	13.95	4.52	0.28	6.18	0.29	0.35	-0.44	1.14	
	6[k]	5.44	0.04	0.79	5.90	0.00	0.00	0.04	-0.45	-0.14	0.12	
	7	5.61	0.04	0.72	5.57	0.09	1.61	0.44	0.05	-0.14	0.14	
	8	6.42	0.34	5.33	6.65	0.08	1.21	0.14	-0.22	-0.34	0.29	
	9	6.71	0.48	7.15	6.75	0.11	1.61	0.85	-0.04	-0.16	0.16	

[a]Each contamination level represents a separate lot of the naturally contaminated food matrix. Enough levels were tested to achieve contamination levels across a 3 Log_{10} range.
[b]Mean of 5 replicate portions, after logarithmic transformation: Log_{10}[CFU/g + (0.1)f].
[c]Repeatability standard deviation.
[d]Relative standard deviation for repeatability.
[e]P-value for a 2-tail t-test, p-value <0.05 indicates significance at the 95% confidence level.
[f]Mean difference between the candidate and reference methods.
[g]Confidence interval.
[g]95% Lower confidence limit for difference of means.
[h]95% Upper confidence limit for difference of means.
[i]Square of the correlation coefficient.
[j]Silliker Laboratory samples.

REFERENCES CITED

1. Meighan, P., Validation of the MicroSnap Total for the Enumeration of Aerobic Heterotrophic Bacteria in Selected Foods, AOAC® *Performance Tested*℠ certification number 031501.
2. AOAC Research Institute Validation Outline for MicroSnap Total for the Enumeration of Aerobic Heterotrophic Bacteria, Approved – March 2015.
3. ISO 4833:2003, Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of microorganisms – Colony-count technique at 30°C
4. AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces Official Methods of Analysis of AOAC INTERNATIONAL, 19th Ed., Appendix J, AOAC INTERNATIONAL, Gaithersburg, MD, http://www.eoma.aoac.org