Method Comparison Study Report for the ISO 16140-2:2016 validation of SyLAB AMP6000 TMAC, for the enumeration of Total mesophillic aerobic count in foods

MicroVal study number: 2015LR60

Method/Kit name: SyLAB AMP6000 TMAC

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Foreword

This report is prepared in accordance with ISO 16140-2:2016 and MicroVal technical committee interpretation of ISO 16140-2 v.1.0

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Method/Kit name: SyLab AMP6000 TMAC

Validation standard: ISO 16140-2:2016 Microbiology of the food chain —Method validation —Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method

Reference methods: ISO 4833-1:2013 Microbiology of the food chain — Horizontal method for the enumeration of microorganisms Part 1: Colony count at 30 degrees C by the pour plate technique

Scope of validation: Raw meats, Fruit and Vegetables and Multi-Component foods

Certification organization: MicroVal

List of abbreviations

- AL Acceptability Limit
- AP Accuracy Profile
- Art. Cont. Artificial contamination
- CFU Colony Forming Units
- CL confidence limit (usually 95%)
- EL Expert Laboratory
- $\bar{D}$ Average difference
- g Gram
- h Hour
- ILS Interlaboratory Study
- Inc/Ex Inclusivity and Exclusivity
- LOQ Level of Quantification
- MCS Method Comparison Study
- min minute
- ml Millilitre
- MR (MicroVal) Method Reviewer
- MVTC MicroVal Technical Committee
- EL Expert Laboratory
- n number of samples
- na not applicable
- neg negative (target not detected)
- NG no growth
- nt not tested
- RT Relative Trueness
- SD standard deviation of differences
- 10^{-1} dilution 10-fold dilution of original food
- 10^{-2} dilution 100-fold dilution of original food
- PCA Plate Count Agar
- MPN Most Probable Number
- BPW Buffered Peptone Water

Contents

1 Introduction

In this project a MicroVal validation study, based on ISO 16140-2:2016, of alternative method(s) for the enumeration of Total Mesophillic Aerobic Count in three different food categories was carried out by Campden BRI as the MicroVal Expert Laboratory.

The reference method used is: ISO 4833-1:2013 Microbiology of the food chain — Horizontal method for the enumeration of microorganisms Part 1: Colony count at 30 degrees C by the pour plate technique.

Scope of the validation study is: Three categories of foods.

Categories included:

- Raw meats,
- Fruit and Vegetables
- Multi-Component foods

Criteria evaluated during the study have been:

- Relative trueness study;
- Accuracy profiles;
- Interlaboratory Study

The final conclusion on the Method Comparison study and ILS is summarized below:

The alternative method SyLAB AMP 600 TMAC, shows comparable performance to the reference method ISO 4833-1:2013 Microbiology of the food chain — Horizontal method for the enumeration of microorganisms Part 1: Colony count at 30 degrees C by the pour plate technique for the enumeration of Total Mesophillic Aerobic Count in Raw meats, Fruit and Vegetables, Multi-Component foods and is restricted to these validated categories.

2 Method protocols

The Method Comparison Study was carried out using 10g gram portions of sample material.

According to ISO 16140-2 the reference method and alternative methods were performed with the same sample. The study was therefore a paired study design.

2.1 Reference method

See the flow diagram in Annex A.

Sample preparations used in the reference method were done according to ISO 6887-series (parts 1,2) and ISO 4833-1:2013 for Raw meats, fruits and vegetables and multicomponent foods.

2.2 Alternative method

See the flow diagram of the alternative method in Annex B.

See the SyLab AMP6000 TMAC kit insert in Annex C.

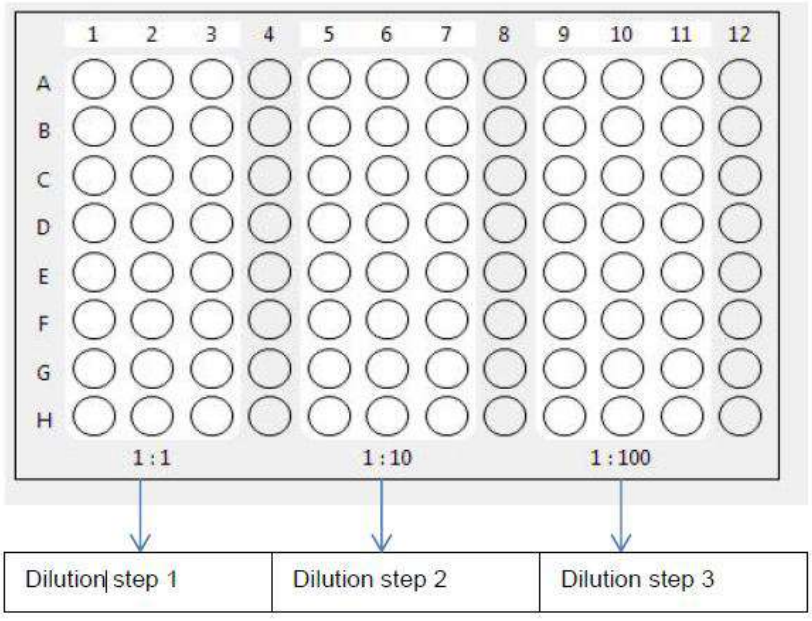
The alternative method principle is based on on a miniaturised automated most probable number method.

The AMP-6000 TMAC analysis system is a platform for determining the mesophilic aerobic colony count by means of a miniaturized and automated MPN ("Most – probable – number") system which requires minimal sample preparation to achieve a quantitative result within 44- 48 h.
The system consists of the "Automated Pipetting System" (AMP – 6000 APS), which is used for processing samples on microtiter plates (96 wells) and a scanner including an evaluation software (AMP – 6000 LabImager TR) for the evaluation of the plates.

The method determines the "most probable number of microbes" by examining multiple replicates of several sample dilutions through statistical means using 24 wells for each dilution (Figure 1). Samples of foods containing mesophilic aerobic organisms are grown in a non-selective TMAC medium.

Figure 1: Format of microtiter plate

Figure 1: Format of microtiter plate



During the evaluation of the individual wells, the colour changes (from orange to yellow and/or from orange to red) and turbidity are detected by measuring reflection. The "most probable bacterial count" is calculated

by means of the software. The use of 24 replicates per dilution step enables a very high precision of the statistical calculation.
The food samples were prepared for analysis and diluted in accordance with ISO 6887. Generally, the diluent used was peptone salt diluent. Dried products were soaked for an hour before analysis and high fat products (>20%) were diluted BPW with added Tween in accordance with ISO 6887.

2.3 Study design

Samples of product were diluted 1 in 10 with an appropriate diluent according to ISO 6887 and homogenised in a stomacher. Appropriate serial dilutions were made and all relevant dilutions were analysed using the reference method. An appropriate dilution was selected as the initial dilution for the alternative method following the manufacturers instructions.

3 Method comparison study

3.1 Relative trueness study

The trueness study is a comparative study between the results obtained by the reference method and the results of the alternative method. This study was conducted using naturally contaminated samples. Different categories, types and items were tested.

A total of 3 categories were included in this validation study. A minimum of 15 items for each category were tested by both the reference method and the alternative method in the relative trueness study, with a minimum of 15 interpretable results per category.

3.1.1 Number of samples
The categories, the types and the number of samples analyzed are presented in Table 1.

Table 1 – Categories, types and number of samples analyzed

Category	Types	Number samples analysed	Number of interpretable results
Fresh produce and fruits	Cut ready to eat fruit e.g. fruit mixes, highly coloured fruit mixes e.g. red/blue berries	5	5
	Cut ready to eat vegetables e.g. Bagged pre-cut salads and shredded carrot, red cabbage	5	5
	Leafy greens/Sprouts e.g. soy, mung, alfalfa,	5	5
	Total	15	15
Raw and ready to cook meat and poultry	Unprocessed Poultry: e.g.carcasses	5	5
	Ready to cook e.g. frozen burgers, patties	6	6
	Raw meats: mince, sausages, whole cuts	6	6

Category	Types	Number samples analysed	Number of interpretable results
	Total	17	17
Multi component foods or meal components	Ready to re-heat refrigerated food e.g. cooked chilled foods, rice and pasta, products, curry	5	5
	Ready to re-heat food frozen e.g. fries, pizza	5	5
	Composite foods with substantial raw ingredients e.g.pasta salads, sandwiches	5	5
	Total	15	15

47 samples were analysed, leading to 47 exploitable results.

3.1.2 Test sample preparation

Naturally contaminated samples were analysed for this trial.

3.1.3 Protocols applied during the validation study

The Alternative method has two different protocols that can be applied, these were included in the validation study, as described below:

Test Protocol	*Application*	*Study*
SY-TMACnT	Standard protocol (44 – 48h) Applicable for samples with strong turbidity,	Included in relevant turbid samples, detailed in results tables in appendices
SY-TMAC	Standard protocol (44 – 48h) Applicable for samples with no turbidity,	Included in all non-turbid samples, detailed in results tables in appendices

The plates for the reference method were incubated for the minimum time of 69 hours.

The microtitre plates for the alternative method were incubated for the minimum time of 44 hours.

Confirmations if required for the alternative method

No confrimation steps were required in this study.

3.1.4 Test results

3.1.5 Calculation and interpretation of relative trueness study

The obtained data were analyzed using the scatter plot. The graphs are provided with the line of identity (y = x).

Figure 2 - Scatter plot of the reference method versus alternative method results for the Fresh produce category.

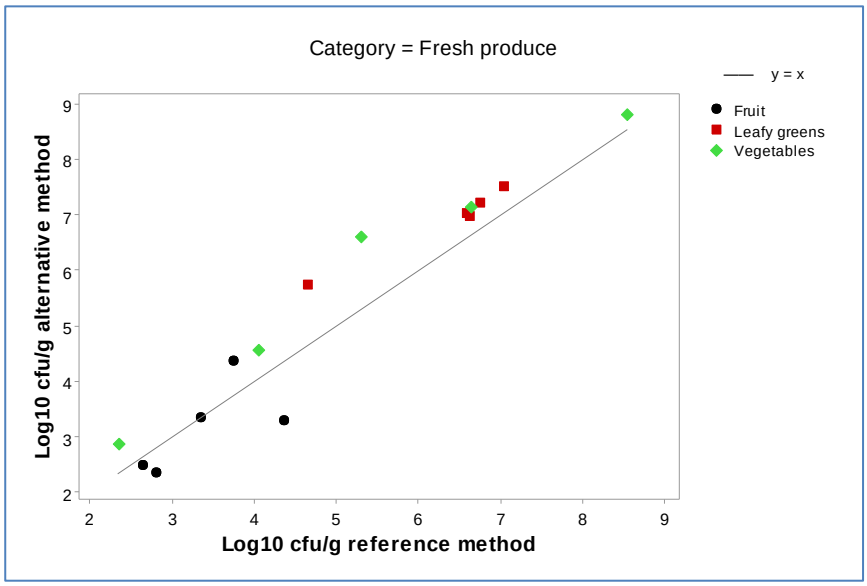


Figure 3- Scatter plot of the reference method versus alternative method results for the Multi Component foods.

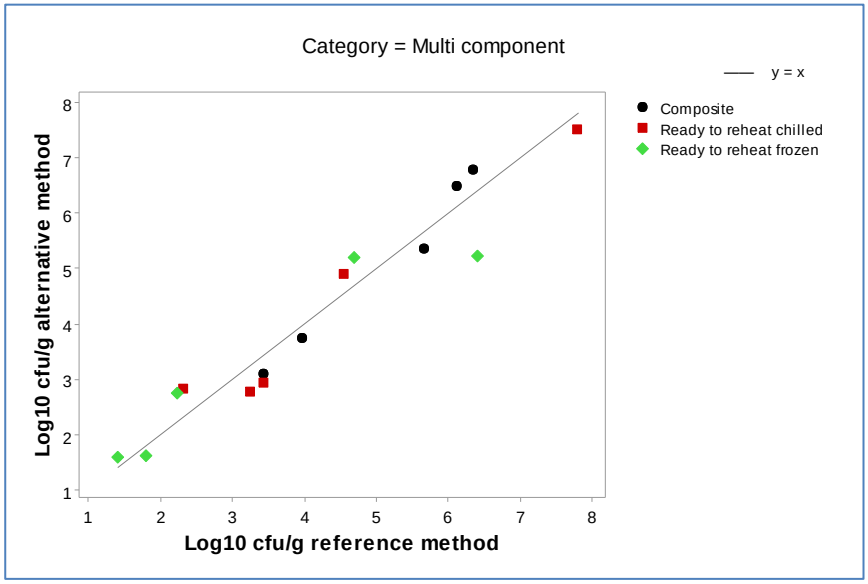


Figure 4- Scatter plot of the reference method versus alternative method results for the Meat category.

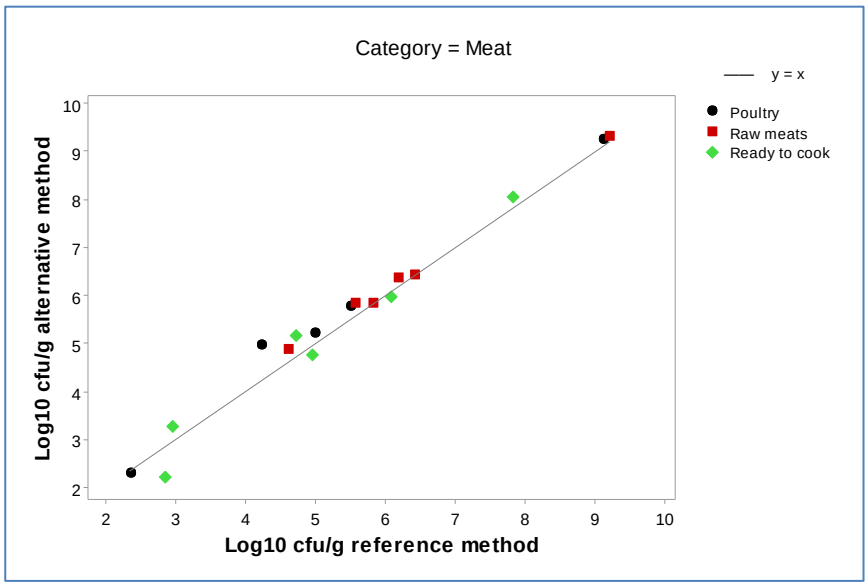
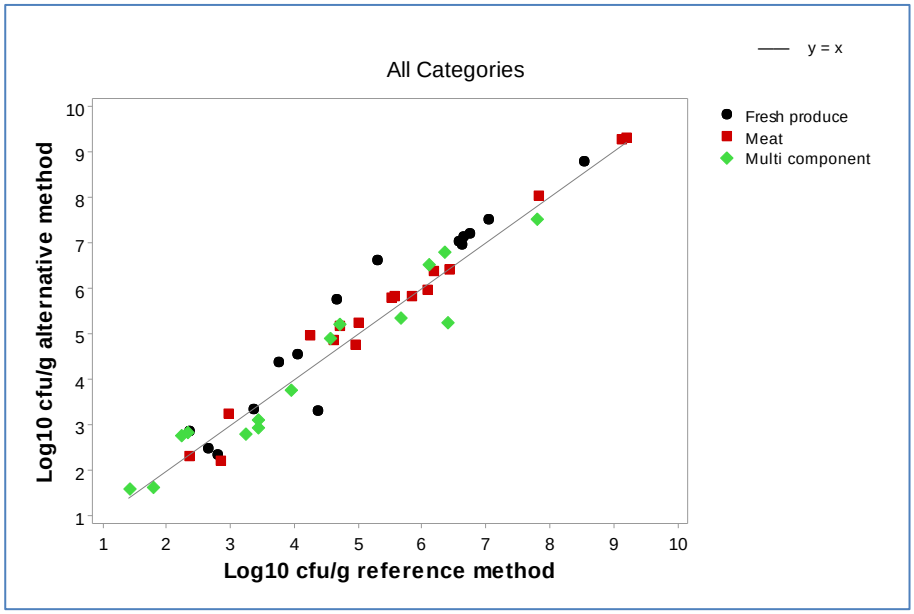


Figure 5 - Scatter plot of the reference method versus alternative method results for all the categories.



According to ISO16140-2:2016 6.1.2.3, the results of the scatter plot are interpreted on the visual observation of the amount of bias and extreme results. The data appears generally acceptable, however the fruit and vegetable category shows a slight positive bias for the alternative method.

The average difference $\overline{D}$, the standard deviation of difference s_D and the limits of agreement were calculated per category and for all categories, this is shown in Table 2.

Table 2- Summary of the calculated values per category

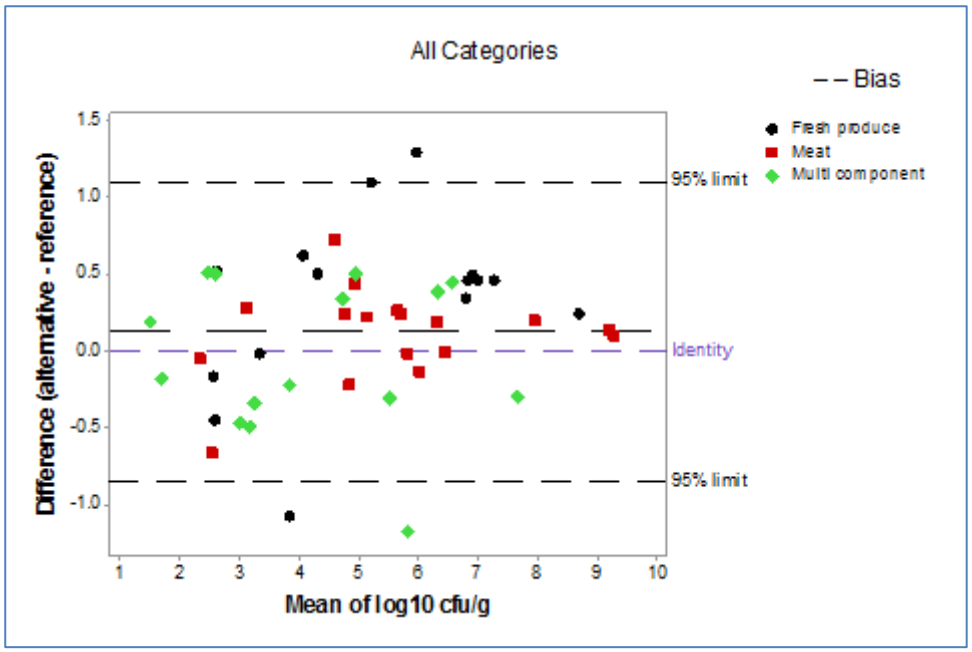
Category	n	$\overline{D}$	s_D	95% Lower limit	95% Upper limit
Fruit and Vegetables	15	0.316	0.580	-0.968	1.600
Multi component foods	15	0.114	0.297	-0.535	0.763
Raw Meats	17	-0.043	0.498	-1.147	1.061
All Categories	47	0.128	0.479	-0.847	1.103

$\overline{D}$: Average difference SD: standard deviation of differences n: number of samples

The data in Table 2 reflect the observations from Figure 2, that results with fruit and vegetable products produce a larger range than the other products, however the bias for this group as a whole is <0.5 log. This is also true for all the categories. Overall there was a slight positive bias in the "all categories" data.

The individual sample differences were plotted against the mean values on a graph, that shows the line of identity (zero difference), the line of bias, and the upper and lower 95% confidence limits of agreement of the bias. Although the text specifies four lines, the example in 16140 Figure 3 shows only three. We have plotted the "line of bias" at $\overline{D}$ as well as the line of identity and confidence limits. This Bland-Altman difference plot for all the samples is given Figure 6.

Figure 6 – Bland-Altman difference plot for all the samples



Samples for which the difference between the result observed with the reference and the alternative methods is above or lower than the limits are listed in the Table 3.

Table 3 - Data which are outside of the accepted limits

Food Category	Food type	Sample code	Food item	Reference method Log cfu/g	Alternative method Log cfu/g	Mean	Difference log cfu/g (alternative – reference)
Fresh Produce	Ready to eat vegetables	17	Cauliflower & Broccoli florets	5.301	6.588	5.944	1.29
Fresh Produce	Ready to eat fruit	122	Tropical fruit shaker	4.362	3.281	3.821	-1.08
Multi-component foods	Ready to reheat frozen	18	Thin pepperoni pizza	6.398	5.217	5.808	-1.18

For “all categories” there are 3 in 47 data values which lie outside the CLs (All categories plot). This is in line with the expectation of not more than one in 20 (i.e .not more than 3 in 60). There were no identifiable trends in these data and they covered 2 of the 3 food categories. The fresh produce sample that is set on the CL line, has a value of 1.095 which is just within the confidence limit of 1.103. These outlier samples were all the same ones as identified in the scatter plots.

3.1.6 Conclusion (RT study)

The relative trueness of the Alternative method is satisfied, as the expectation of not more than 1 in 20 data points outside of the acceptability limits is met.

3.2 Accuracy profile study

The accuracy profile study is a comparative study between the results obtained by the reference and the results of the alternative method. This study is conducted using artificially contaminated samples, using one type per category.

3.2.1 Categories and sample types

Three food categories were tested with two different batches of a single food type, using 6 samples per type. Two samples were contaminated at a low level, 2 at intermediate level, 2 at a high level. To obtain each level highly contaminated food for each category and each batch were mixed to produce the “High”, “Medium” and “Low” levels. For each sample, 5 replicates (5 different test portions) were tested. A total of 30 samples were analysed per food type. The following food type pairs were studied (See Table 4):

Table 4 - Categories, types, items, strains and inoculation levels for accuracy profile study

Category	Types	Item	Samples/Target Level*	Test portions
Fruits and vegetables	Fresh produce	Shredded red cabbage (To test highly coloured foods)	Low 100cf/g	5
			Low 100cf/g	5
			Medium : 50.00cfu/g	5
			Medium : 50.00cfu/g	5
			High : 5.000.00cfu/g	5
			High : 5.000.00cfu/g	5
Raw poultry and meats	Fresh meat	Pork mince	Low 1000cf/g	5
			Low 1000cf/g	5
			Medium : 50.000cfu/g	5
			Medium : 50.000cfu/g	5
			High : 1.000.000cfu/g	5
			High : 1.000.000cfu/g	5
Multi component foods	Composite foods with raw ingredients	Cooked chilled rice	Low 100cf/g	5
			Low 100cf/g	5
			Medium : 50.000cfu/g	5
			Medium : 50.000cfu/g	5
			High : 1.000.000.00cfu/g	5
			High : 1.000.000.00cfu/g	5

*Target only, levels may vary due to samples being naturally contaminated

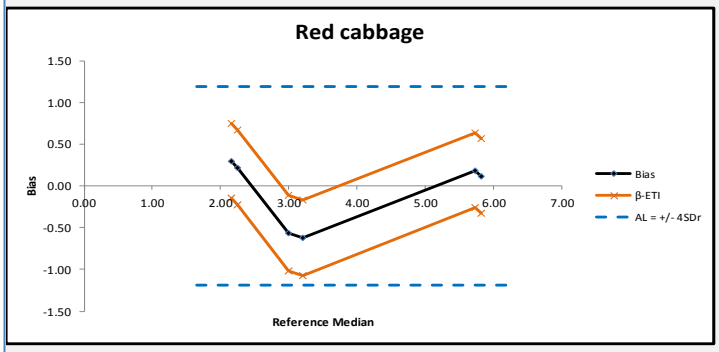
3.2.2 Calculations and interpretation of accuracy profile study

The statistical results and the accuracy profiles are provided Figures 7 to 9.

The calculations were done using the AP Calculation Tool MCS (Clause 6-1-3-3 calculation and interpretation of accuracy profile study) available on http://standards.iso.org/iso/16140

Figure 7 – Accuracy profile –Category fresh produce (type red cabbage)

(Food) Category	Produce
(Food) Type	Red cabbage

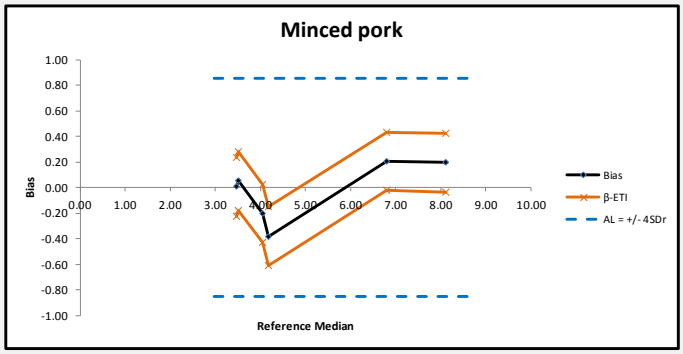


Sample Name	Reference central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
91	2.16	0.298	-0.151	0.747	NO	YES
114	2.25	0.220	-0.229	0.669	NO	YES
263	3.00	-0.565	-1.014	-0.116	NO	YES
41	3.20	-0.622	-1.071	-0.173	NO	YES
79	5.72	0.186	-0.263	0.635	NO	YES
171	5.81	0.117	-0.332	0.565	NO	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.297	0.311	NO	+/- 1.188

Figure 8 – Accuracy profile – Category raw meat and poultry (type minced pork)

(Food) Category	Meat
(Food) Type	Minced pork

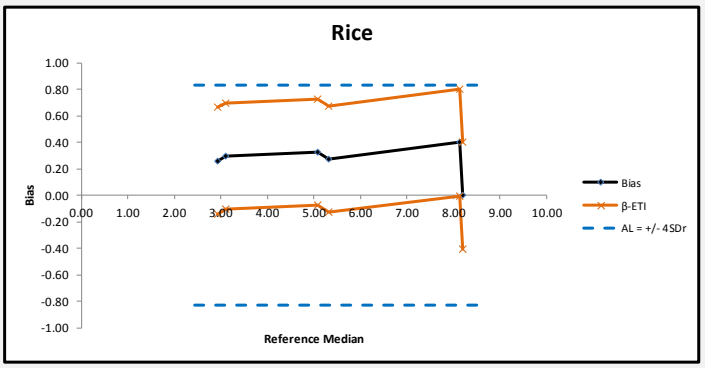


Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
77	3.48	0.006	-0.224	0.235	YES	YES
212	3.51	0.052	-0.177	0.282	YES	YES
66	4.04	-0.203	-0.433	0.026	YES	YES
225	4.18	-0.383	-0.613	-0.153	NO	YES
80	6.81	0.204	-0.025	0.434	YES	YES
147	8.11	0.194	-0.036	0.423	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.214	0.159	NO	+/- 0.856

Figure 9 – Accuracy profile – Category multi component foods (type chilled rice)

(Food) Category	Multi
(Food) Type	Rice



Sample Name	Reference Central value	Bias	Lower β-ETI	Upper β-ETI	β-ETI compared to AL=±0.5 Acceptable	β-ETI compared to final AL Acceptable
296	2.93	0.260	-0.141	0.661	NO	YES
188	3.11	0.294	-0.107	0.696	NO	YES
122	5.08	0.327	-0.074	0.729	NO	YES
54	5.32	0.271	-0.130	0.672	NO	YES
208	8.15	0.398	-0.003	0.799	NO	YES
17	8.20	-0.003	-0.404	0.399	YES	YES

	Reference method	Alternative method	SD repeatability of reference method <= 0.125	Final AL
SD Repeatability	0.207	0.278	NO	+/- 0.828

- For all of the categories, the *S*ref was >0,125, and one or more of the upper or lower limits were greater than 0.5 log, therefore the new ALs calculation was carried out.

- For the fruits and vegetables category, there were originally 6 out of 12 limits exceeded and the *S*ref was 0.297. This gave new calculated ALs of 1.188, this calculation is based on the variation in counts from the reference method, this high AL demonstrates variation in levels between the replicate samples and is due to the sample type. All data points were within these limits

- For Raw meats, there were originally 1 out of 12 limits exceeded and the *S*ref was 0.214. This gave new calculated ALs of 0.856 and all data points were within these limits

- For Multicomponent foods, there were originally 5 out of 12 limits exceeded and the *S*ref was 0.207. This gave new calculated ALs of 0.828 and all data points were within these limits.

The accuracy of the Alternative method is satisfied as the all categories met the re-calculated AL

There was a slight positive bias for the Multicomponent food group, in this study but not in the fresh produce group as observed in the relative trueness study.

All the accuracy profiles fulfil the performance criteria and the alternative method is accepted as being equivalent to the reference method, for the categories tested, although it should be noted that the newly calculated AL's were quite large between (0.825 and 1.18)

3.3 Inclusivity / exclusivity

Inclusivity and exclusivity testing is not required for general enumeration methods such as total plate count (TPC) and yeast & mould (Y&M) methods. Inclusivity and exclusivity testing has **not been done** for the alternative method in this study

3.4 Limit of quantification (LOQ)

The LOQ is only relevant when the measurement principle of the alternative method is not based on counting visible colonies of the target microorganism, i.e. it applies only to instrumental methods. As the AMP-6000 is an instrumental method then according to ISO/FDIS 16140-2:2015 it should be tested for LOQ. However, in this case, the AMP-6000 TMAC is a non-selective method for total bacterial counts and it is not possible to have blank samples to test as would be the case for selective methods. Therefore, it was agreed at the proposal stage not do the LOQ determination as it would not be possible to have blank food samples.

4 Interlaboratory study

The inter-laboratory study is a trial performed by multiple laboratories testing identical samples at the same time. The results of which are used to estimate alternative-method performance parameters.

4.1 Study organisation

4.1.1 Collaborators
Samples were sent to 5 laboratories; 2 collaborators were involved in the study for all the laboratories Matrix

Naturally contaminated pork mince was used for this study. Pork mince was divided into batches and stored at 6 temperature/ time combinations, (-18^{0}C, 22^{0}C for 3 & 24hours, 30^{0}C for 3, 5 & 24hour) enumerated using the reference method and stored at -18^{0}C. This was done to produce samples of different contamination levels to allow the three contamination levels to be prepared.

4.1.2 Sample preparation
Samples were prepared on Friday 16th November 2017 as described below:

The samples were defrosted overnight and mixed to obtain the desired levels. For each collaborator, a set of samples was prepared containing 2 samples at a low level, two samples at a medium level, two samples at a high level and a single uninoculated blank sample (tinned minced beef was used for the blank sample). The samples were blind-coded so that the collaborators did not know the intended contamination level. As the laboratories had two different collaborators, a different set of codes were used for each collaborator. A

set of samples was also prepared for the EL, although the data from these was not used in the data analysis. Five samples at each level were also kept for homogenity analysis described in 4.2.2.

Once prepared the samples were stored frozen (-18ºC).

The target levels and codes are shown below

Table 5: Contamination levels

Contamination level	Sample code Collaborator 1	Sample code Collaborator 2
Uninoculated	[4]	[8]
Low (10^3 cfu/g)	[1]	[13]
Low (10^3 cfu/g)	[5]	[14]
Medium (10^5 cfu/g)	[2]	[10]
Medium (10^5 cfu/g)	[6]	[12]
High (10^7 cfu/g)	[3]	[9]
High (10^7 cfu/g)	[7]	[11]

4.1.3 Labelling and shipping
Blind coded frozen samples were placed in isothermal boxes, which contained cooling blocks, and express-shipped to the manufacturer laboratory on Monday 20th November. The samples were stored frozen (-18ºC), and shipped to participating laboratories on Monday 27th November.

A temperature control flask containing a sensor was added to the package in order to register the temperature profile during the transport, the package delivery and storage until analyses.

Samples were shipped within 24 h to the involved laboratories. The temperature conditions had to stay lower or equal to 8°C during transport, and allowed to thaw between 0°C – 8°C in the labs.

4.1.4 Analysis of Samples
Collaborative study laboratories and the expert laboratory carried out the analyses on Tuesday 28th November with the alternative and reference methods. The analyses by the reference method and the alternative method were performed on the same day.

4.2 Experimental parameters controls

4.2.1 Detection of target organisms in the matrix before inoculation
The samples were naturally contaminated therefore this step was not required.

4.2.2 Sample variation
For each level 5 samples were stored at -18±3^{0}C for 7 weeks and then tested using the reference method. As samples were delivered frozen it was not necessary to determeine stability on during chill storage

The results are summarized in Table 6, the variation in levels between samples was very low.

Table 6 – Homogeneity of levels of Total Mesophillic Aerobic Count between 5 samples at each level

Level	replicate	Reference Method	
		cfu/g	log cfu/g
High	1	3.3E+07	7.52
	2	3.9E+07	7.59
	3	2.5E+07	7.40
	4	2.5E+07	7.40
	5	4.1E+07	7.61
	Mean	**3.3E+07**	**7.51**
	stdev		**0.10**
Medium	1	1.1E+05	5.04
	2	1.6E+05	5.20
	3	1.6E+05	5.20
	4	1.1E+05	5.04
	5	8.6E+04	4.93
	Mean	**1.3E+05**	**5.10**
	stdev		**0.12**
Low	1	6.2E+03	3.79
	2	1.1E+04	4.04
	3	6.0E+03	3.78
	4	5.6E+03	3.75
	5	6.8E+03	3.83
	Mean	**7.1E+03**	**3.85**
	stdev		**0.12**
Blank	1	<10	<1
	2	<10	<1
	3	<10	<1
	4	<10	<1
	5	<10	<1
	Mean	**<10**	**<1**
	stdev		**0**

4.2.3 Logistic conditions

The temperatures were measured at receipt by the collaborators, the temperatures registered by the thermo-probe, and the receipt dates are given in Table 7.

Table 7 - Sample temperatures at receipt

Collaborator	Average Temperature measured by the probe (°C)	Temperature measured at receipt (°C)	Receipt date and time	Analysis date
1	-16	Frozen*	27/11/17	27/11/17
2	-15	Frozen*	27/11/17	27/11/17
3	-15	Frozen*	27/11/17	27/11/17
4	-15	Frozen*	27/11/17	27/11/17
5	-15	Frozen*	27/11/17	27/11/17

*The water was frozen and therefore the temperature could not be established with a thermometer.

No problem was encountered during the transport or at receipt for the 5 collaborating laboratories. All the samples were delivered on time and in appropriate conditions. Temperatures during shipment and at receipt were all correct.

4.3 Calculation and summary of data

4.3.1 MicroVal Expert laboratory results

The results obtained by the expert laboratory are given in Table 8.

Table 8 – Results obtained by the expert lab.

Level	Reference method (Log cfu/g)	Alternative method (Log cfu/g)
Blank	<1.00	<1.28
Low	3.59	3.64
Low	3.46	3.75
Medium	4.90	5.16
Medium	4.90	5.64
High	7.18	7.36
High	7.36	7.59

4.3.2 Results obtained by the collaborative laboratories

The data from the collaborative trial were calculated and interpreted according to section 6.2.3 of ISO 16140-2:2016 using the freely available Excel® spreadsheet (http://standards.iso.org/iso/16140). Version 14-03-2016 was used for these calculations.

The results obtained by the collaborators are shown in Table 9.

The accuracy profile plot is shown in Figure 10 and the statistical analysis of the data shown in Table 10.

Table 9: Summary of the results of the interlaboratory study per analyte level

Laboratory	Collaborator	Level	Reference method (Log cfu/g)		Alternative method (Log cfu/g)	
			Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
1	1	low	3.87	3.87	3.54	3.99
1	2	low	3.69	3.78	3.67	3.71
2	3	low	3.91	3.88	3.76	3.64
2	4	low	3.81	4.15	3.95	3.95
3	5	low	3.70	3.62	3.80	3.47
3	6	low	3.61	3.65	3.76	3.91
4	7	low	3.91	4.23	3.53	3.57
4	8	low	4.04	3.79	3.74	3.50

Laboratory	Collaborator	Level	Reference method (Log cfu/g)		Alternative method (Log cfu/g)	
5	9	low	4.04	3.52	3.56	3.59
5	10	low	3.92	3.86	3.40	4.11
1	1	medium	5.28	5.23	5.37	5.36
1	2	medium	5.20	5.20	5.03	5.20
2	3	medium	5.68	5.19	5.45	5.02
2	4	medium	5.40	5.30	5.17	5.16
3	5	medium	5.18	5.18	5.35	5.45
3	6	medium	5.15	5.23	5.05	5.20
4	7	medium	5.44	5.80	5.59	6.02
4	8	medium	5.28	5.60	5.20	5.55
5	9	medium	5.30	5.23	5.50	5.20
5	10	medium	5.23	5.11	5.31	5.17
1	1	high	7.41	7.40	7.74	7.69
1	2	high	7.34	7.45	7.74	7.41
2	3	high	7.46	7.64	7.24	7.69
2	4	high	7.41	7.61	7.20	7.37
3	5	high	7.47	7.56	7.64	7.84
3	6	high	7.45	7.59	7.55	7.20
4	7	high	7.73	8.08	8.09	8.20
4	8	high	7.57	7.57	7.59	7.45
5	9	high	7.28	7.45	7.34	7.34
5	10	high	7.30	7.32	7.25	7.45
1	1	blank	<1.00		1.58	
1	2	blank	<1.00		<1.28	
2	3	blank	<1.00		1.58	
2	4	blank	<1.00		1.78	
3	5	blank	<1.00		<1.28	
3	6	blank	0.70		<1.28	
4	7	blank	<1.00		1.28	
4	8	blank	<1.00		<1.28	
5	9	blank	<1.00		2.28	
5	10	blank	<1.00		2.16	

Figure 10. Accuracy profile of SyLAB AMP6000 TMAC from the ILS

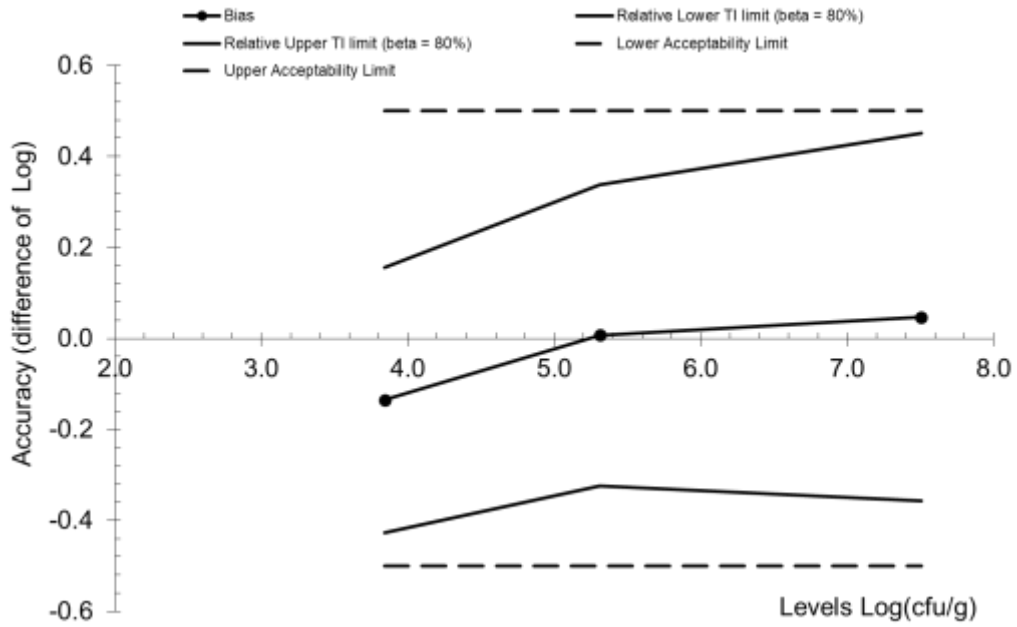


Table 10. Statistical analysis of the ILS data according to the ISO spreadsheet

Accuracy profile						
Study Name	SyLab TMAC					
Date	28/11/2017					
Coordinator	Campden BRI					
Tolerance probability (beta)	80%	80%	80%			
Acceptability limit in log (lambda)	0.50	0.50	0.50			

FALSE

Application of clause 6
Step 8: If any of the values for the β
the acceptability limits, calculate th
reproducibility standard deviation
method.
Step 9: Calculate new acceptabi
function of this standard de

	Alternative method			**Reference method**		
Levels	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
Target value	3.843	5.311	7.505			
Number of participants (K)	10	10	10	10	10	10
Average for alternative method	3.708	5.318	7.552	3.843	5.311	7.505
Repeatability standard deviation (sr)	0.214	0.181	0.170	0.168	0.161	0.114
Between-labs standard deviation (sL)	0.000	0.156	0.232	0.073	0.095	0.145
Reproducibility standard deviation (sR)	0.214	0.239	0.287	0.183	0.186	0.184
Corrected number of dof	18.947	15.456	12.688	18.197	17.315	13.113
Coverage factor	1.361	1.386	1.407			
Interpolated Student t	1.328	1.339	1.352			
Tolerance interval standard deviation	0.2193	0.2471	0.2990			
Lower TI limit	3.417	4.987	7.147			
Upper TI limit	3.999	5.649	7.956			
Bias	**-0.135**	**0.007**	**0.047**			
Relative Lower TI limit (beta = 80%)	**-0.427**	**-0.324**	**-0.358**	FALSE		
Relative Upper TI limit (beta = 80%)	**0.156**	**0.338**	**0.451**	FALSE		
Lower Acceptability Limit	**-0.50**	**-0.50**	**-0.50**			
Upper Acceptability Limit	**0.50**	**0.50**	**0.50**			

Select ALL blue lines to draw the accuracy profile as illustrated in the worksheet "Graph Profile"

New acceptability limits may be based on reference method pooled variance	
Pooled repro standard dev of reference	0.185

5 Overall conclusions of the validation study

- The alternative method SyLab AMP 6000 TMAC for enumeration of Total Mesophillic Aerobic Count shows satisfactory results for relative trueness.
- The alternative SyLab AMP 6000 TMAC for enumeration of Total Mesophillic Aerobic Count shows satisfactory results for accuracy profile.
- The alternative SyLab AMP 6000 TMAC for enumeration of Total Mesophillic Aerobic Count shows satisfactory performance in the ILS.
- The alternative SyLab AMP 6000 TMAC for enumeration of Total Mesophillic Aerobic Count shows comparable performance to the reference method ISO 4833-1:2013 Microbiology of the food chain — Horizontal method for the enumeration of microorganisms Part 1: Colony count at 30ºC by the pour plate technique.

Date 29/03/2019

Signature

Annexes

A. Flow diagram of the reference method
B. Flow diagram of the alternative method
C. Test kit insert

ANNEX A: Flow diagram of the reference method

Food sample (10g) + appropriate diluents (90ml) according to ISO 6887

Homogenise and dilute further as required

↓

Plate 1ml samples of appropriate dilutions and pour with tempered PCA

↓

Incubate at 30 ± 1°C

for 72h±3h

(The minimum of 69h was used)

↓

Count all colonies

↓

Calculate cfu/g

ANNEX B: Flow diagram of the alternative method

Food sample (10g) + appropriate diluents (90ml) according to ISO 6887

Homogenise and dilute further as required

↓

Choose working dilution to test based on likely contamination level*

↓

Place diluted sample, media and 96 well microtitre plate labelled with the provided bar code into the AMP-6000. Plate is automatically filled with 180ul of TMAC media and 20ul of sample (neat, 1:10 or 1:100 dilution)

↓

Remove filled microtitre plate and seal. Incubate at 30 ± 1°C for 44 – 48h

(The minimum of 44h was used)

↓

Place microtitre plates in the AMP-6000 and evaluate. Positive colour changes (from orange to yellow or red) and turbidity is automatically read.

↓

The TMAC per sample will be automatically calculated as cfu/g after 44h

ANNEX C: Kit insert(s)

Standardized report - Quantitative methods - Method Comparison Study and ILS 2015LR60 SYLAB AMP6000 TMAC Version 2 SUMMARY REPORT

AMP – 6000

Determination of Total Mesophilic Aerobic Count

Application Manual, Version 1.2.2, October 2018
Product Number 62-100323
60 Tests

SY – LAB Geräte GmbH
Tullnerbachstr. 61 – 65
A – 3011 Neupurkersdorf

CONTENT

1. INTRODUCTION

The increasing importance of a comprehensive approach to quality in the food production leads to quality indicators such as total mesophilic aerobic count (TMAC) play an increasingly important role in the control of the hygienic and commercial quality of food. Since the conventional method (Reference method according to EN ISO 4833) is complicated and time – consuming to carry out, alternative methods are preferred, which allow rapid, automated and reliable determination of hygiene parameters and simplifying the documentation.

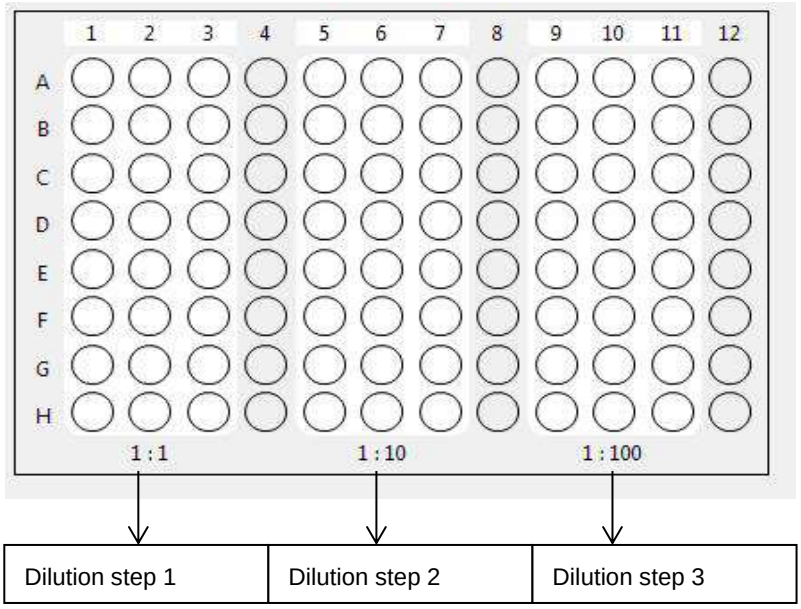
2. INTENDED USE & SCOPE

The AMP – 6000 Analysis System is a platform for enumeration of the aerobic colony count by means of a miniaturized and automated MPN (*"Most – probable – number"*). ***The scope for method validation is specified for the categories "Raw Meat Products", "Fruit & Vegetable Products" and "Multi Component Products"***. Environmental samples are not part of validation nevertheless these samples can be also tested without restriction of any kind. It leads with minimal sample preparation within 44 – 48 hours for quantitative results. The system consists of the "Automated Pipetting System" (AMP – 6000 APS), which is used for processing samples on microtiter plates (96 wells) and a scanner including an evaluation software (AMP – 6000 LabImager TR) for the evaluation of the plates.

3. TEST PRINCIPLE

The method described under item 2 allows determining the "most probable number of microbes" by examining multiple replicates of several sample dilutions through statistical means. Here, each weighing of several wells, using three dilutions, in a well – defined format (24-24-24; refer to Figure 1) on a microtiter plate loaded automatically and fully parallel by using the robotic system.

Figure 1: Format of microtiter plate



Depending on the existing level of contamination a specific distribution of positive and negative wells is then obtained in the replicates. Using statistical calculation, a probable bacterial count within one dilution step of each of the possible distributions of vegetated and non-vegetated wells can be assigned. In the evaluation of the individual wells the color changes (orange – yellow and/or orange – red) and turbidity are detected by measuring reflection and calculates the "most probable bacterial count" by means of the software. The use of 24 replicates per dilution step inserted enables a very high precision of the statistical calculation.

4. REQUIRED MATERIALS AND EQUIPMENT

- Micro pipettes
- Sterile tips
- Stomacher and Stomacher bag (for analyzing solid samples)
- Vials (≥10ml Volume; for preparation of working dilutions)
- Use Ringer's solution, peptone water, buffered peptone water or other appropriate diluents according to ISO 6887
- AmpMedia 003 (*Ready to use*)
- Microtiter plates
- Sterile sample reservoir
- Sterile medium reservoir
- Tips for robotic system
- AMP – 6000
- Bar codes
- Alternatively, multichannel pipette with adequate tips (for manual preparation of plates)

The AmpMedia 003 has to be stored at 2 – 8°C.

5. PROCEDURE

5.1. Sample Preparation (Day 1)

a. Liquid samples can be used directly without further treatment for the determination of TMAC. The theoretical detection limit for liquid samples is 2 CFU/ml.

b. For solid samples weigh in 10g sample and add 90ml homogenization solution in a sterile stomacher bag by homogenizing the suspension for 1 – 2 minutes. The theoretical detection limit for solid samples is 20 CFU/g.

c. For dried or powdered samples (e.g.: milk powder, baby food, etc.) a regeneration step of the duration of approximately 30 – 60 minutes for stressed or damaged cells must be respected.

d. After sample homogenizing a suitable working dilution (refer to SY – LAB Quick Protocols) has to be prepared, where at the MPN format 24-24-24 covers the following ranges of contamination:

Dilution Steps	*Range of Contamination [CFU]/g product*
D0	**2 – 16,000**
D1	**20 – 160,000**
D2	**200 – 1,600,000**
D3	**2,000 – 16,000,000**
D4	**20,000 – 160,000,000**
D5	**200,000 – 1,600,000,000**
D6	**2,000,000 – 16,000,000,000**

e. The AMP – 6000 APS is started using the toggle switch on the rear panel and the software is opened by entering the password (refer to technical handbook item 3.6.). The device can now be loaded according to the following scheme with the necessary materials:

- *Position R1:* *Sterile 80ml reservoir for medium*
- *Position R2:* *Sterile 80ml reservoir for medium*
- *Position A – D:* *Microtiter plates*
- *Position E:* *Sterile sample reservoir for 4 samples*
- *Position F:* *tip box*

Using the 80ml reservoir on position R2 is only necessary if two applications are processed in parallel. Both reservoirs are empty and have to be filled with medium.

f. Using the sample tracking sheets (see point 11. ANNEX) a bar code is assigned to the sample name. These log sheets provide for each measurement run with 4 plates (samples) a unique set of bar codes corresponding to the positions (A – D) of AMP – 6000 APS sample tables. Then the pre-cut label of the sample tracking worksheets with the plate bar is drawn and glued to the broad side of the right-oriented microtiter plate. Thus, each sample (plate) assigned a unique bar code, with which the sample can be uniquely identified for analysis with the AMP – 6000 LabImager TR. The sample tracking sheets can also simultaneously used as a feed scheme for the AMP – 6000 APS.

g. Using the button "File" (Screen: top left) and then using the "Open" button opens a dialog box where you can select by "Protocol / template" the protocol. **Only specially programmed protocols of the company SY – LAB GmbH are allowed and used for correct processing of the samples in the microtiter plates:**

SY-1 Application 4 Samples
SY-1 Application 3 Samples
SY-1 Application 2 Samples
SY-1 Application 1 Sample
SY-2 Application 2 Samples
SY-2 Application 1 Sample

h. Starting the protocol in the AMP – 6000 APS by pressing the start button software. Here, the pipetting scheme provides that 180µl medium per well are presented in the microtiter plates. Thereafter, 20µl of each sample are added to the first four columns on the plate. Columns 4 and 8 only serve to homogenize and are applied for further dilutions of the sample on the remaining columns 5 – 7 (1:10), respectively, 9 – 11 (1:100). The columns 4, 8 and 12 are not used for evaluation (refer to Figures 1, 2 & 3).

i. Alternatively, the microtiter plates can be loaded in the same way manually using a multichannel pipette for sample dilutions.

j. After this processing cycle of about 12 – 13 minutes, the microtiter plates are removed and sealed with sterile lids. Now, the AMP – 6000 LabImager software can be started. Select the "Create Analysis" to record the sample and label with either barcode scanner or enter manual input. In the next step the following testing protocols can be selected in the field "Test" for the different samples:

Test Protocol	*Application*

SY-TMACnT	Applicable for samples with strong turbidity, which means the crosses (detection tool for evaluation of turbidity), located on the feeder of the AMP – 6000 LabImager TR are not clearly visible.
SY-TMAC	Applicable for samples with no turbidity, which means the crosses located on the feeder of the AMP – 6000 LabImager TR are clearly visible.

Subsequently, the current dilution of the sample preparation must be set in the "Work Dilution" box. The scale samples can be confirmed with the button "Create" and released for scanning. After generating the analysis for the samples, the microtiter plates can be incubated at 30°C for a period of 44 – 48h. The AMP – 6000 APS can be loaded and used for further approaches.

5.2. Sample Evaluation (Day 2)

k. After incubation the microtiter plates are removed from the incubator and brought to room temperature for about 10 – 30 minutes.

l. The AMP – 6000 LabImager TR is put into operation (standby) by turning on the device power switch (rear of device). The scanner is turned on with the toggle switch on the front panel. 4 microtiter plates can be positioned in the depositors of the AMP – 6000 LabImager TR.

m. With the help of the field "Scan Wellplates" the number of plates can be set. Now the plate bar code must be reaffirmed to the sample. The evaluation starts by pressing the button “Next” after a warm-up phase. The detecting of the change in color and the turbidity in the individual wells (see item 6. EVALUATION) is performed by using a reflection measurement.

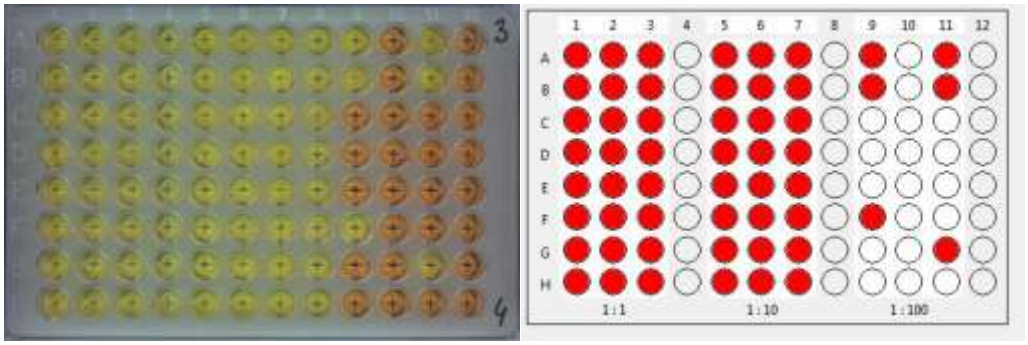
n. The scanned samples (plates) are automatically opened and saved with the "Save" button in the database. This opens a dialog box that is confirmed by "OK" and the results of analysis are shown in a result view.

o. The field "View Analysis" can be opened for further processing already saved projects.

6. EVALUATION

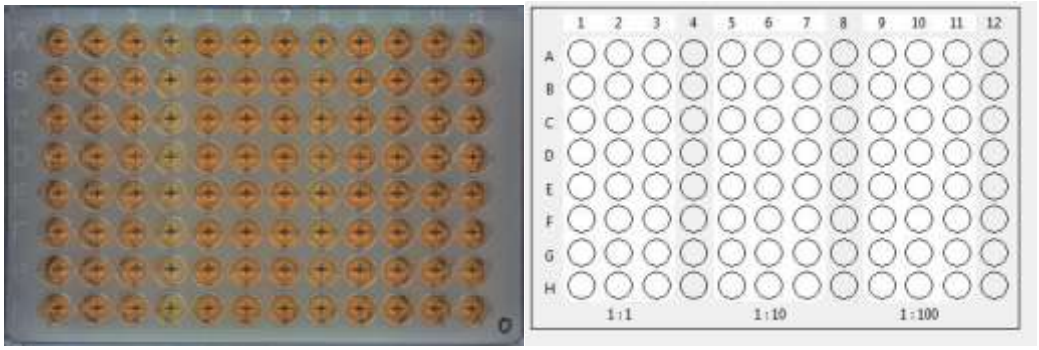
The microtiter plates are scanned and displayed as images. In addition, the change in color in the individual wells is detected by a reflection measurement. If the numerical value of the color in a well is higher than the set cut – off in the test, so this well is rated as positive (color change: orange – yellow and/or orange – red). The second evaluation type is the turbidity of the sample. This dual review of wells results in a combination which is applied calculating the total mesophilic aerobic count in the software by using the Poisson distribution (e.g.: Figures 2 and 3).

Figure 2: Example for correct positive evaluation



Combination: 24-24-6 leads to a final result of 1,92e03 CFU/g sample

Figure 3: Example for correct negative evaluation



Combination: 0-0-0 leads to a final result of ≤ 1,91e00 CFU/g sample

Columns 4, 8 and 12 are not used for evaluation.

7. ACCEPTANCE CRITERIA

When analysing the results, the number of positive wells needs to be considered. The positive well result is given in the format N-N-N, where N = the number of positive wells out of a possible 24 for each microtiter plate dilution series (1:1-1:10-1:100). The following criteria should be applied to ensure reliability:

a) The number of positive wells should follow the microtiter plate dilution series (e.g. 24-22-10). If the number of positive wells do not follow the dilution series (e.g. 7-15-18) the samples need tob e repeated.
b) Where an initial working dilution of 1:100 or greater has been applied (D2 – D6 on page 4), the number of positive wells in the first two microtiter plate dilutions (1:1 and/or 1:10) should not be 0 (e.g. 5-0-0 or 0-0-0). A lower initial working dilution should be used for more reliable results.
c) Where more than one working dilution has to be tested, the result with the highest number of positive wells should be considered the most reliable providing that it does not produce a greater than result. For example if the working dilutions of 1:10, 1:100 and 1:1000 are used and the following results are obtained:

Working Dilution	MPN Index Figure
1:10 (D1)	24-24-24
1:100 (D2)	24-22-10
1:1000 (D3)	12-2-0

In that case the 1:100 (D2) working dilution result should be considered the most reliable, as the 1:10 (D1) working dilution would produce a greater than result.

8. QUALITY CONTROL

Each batch of medium is tested using standardized methods and protocols on the functionality and quality. The release is confirmed and accompanied by a quality control certificate which can always be downloaded from the SY – LAB GmbH website www.sylab.com available).

9. SAFETY INFORMATION

When working with the AMP – 6000 the instruction manual and the enclosed safety data sheets (Available MSDS can always be downloaded from the SY – LAB GmbH website www.sylab.com) of the medium have to be paid attention. The standard safety rules in the laboratory and in the use of chemicals must be identified and met. Never store medium and/or components of medium together with food. Wearing disposable gloves, protective goggles and suitable protective clothing are mandatory. Please take the proper safety precautions (destruction of the contaminated material by disinfection and / or autoclaving) to prevent further contamination.

10. WARRENTY AND LIMITED WARRENTY

SY – LAB GmbH guarantees the functionality and usability of the product for the intended purpose and in the case of the nutrient medium up to the stated expiration date. SY – LAB GmbH assumes no responsibility for consequences (results) or damage whatsoever arising from its use. We also reserve the right to modify the product at any time in order to optimize it. Should any technical problems reported, please do not hesitate to contact our technical service (email: supportbio@sylab.com / Tel.: + 43 – 2231 – 62252 – 0).

11. FAST PROTOCOL

Working Steps	***Duration***
1. Sample Preparation (liquid samples can be used directly and solid samples have to be diluted 1:10)	approx. 5 Min.
2. Processing of samples with AMP – 6000 APS and creating analysis for processed samples in the AMP – 6000 LabImage software simultaneously	approx. 13 Min.
3. Incubation at 30°C	44 – 48h
4. Let the microtiter plates adapt to room temperature	10 – 30 Min.
5. Positioning of microtiter plates (4 plates possible), verifying the barcodes and evaluation by AMP LabImage software	approx. 1 Min.
6. Reading TMAC result and generating report	approx. 1 Min.

12. ANNEX 1: Sample Tracking

AMP–6000 SAMPLE TRACKING SY-LAB

Position	MTP Barcode	MTP Barcode Label	Sample ID	Notes
A	0000001			
B	0000002			
C	0000003			
D	0000004			