MICROBIOLOGICAL FOOD ANALYSIS VALIDATION CERTIFICATE

We hereby certify that the following method and/or products:

The SEILALIMENTOS protocol for the microbiological analysis of foodstuffs (ref. MICROKIT® PRT-SEILA-001, 42 pages, and the derived specifics for each parameter PRT-AL-001 a PRT-AL017), conducted using the described culture medium: **LPT Neutralizing Broth** (Ref. MICROKIT® DMT217, RPL054, TPL053S), **SS Broth** (DMT067, TPL401), **CHROMOSALM Agar** (DMT500, TPL402, RPL012, PPL925), **XLD Agar** (DMT142, TPL504Z), **COMPACT-DRY-PLATES®-SL** (1002973), **B.cereus PREP-MYP Mossel Agar** (DMT015+SAJ001, TPL300+SAJ001, RPL002+SAJ001), **O157 Broth** (BCD165+BCX150, RPL203), **Sorbitol MacConkey Agar** (BCD161+BCX161, RPL025), **MugPlus Cfs.Agar** (DMT400, TPL400, RPL444), **MCC Broth** (DMT900, TPL637), **COMPACT-DRY-PLATES®-EC** (1000168), **Cetrimide Agar** (DMT034, TPL100, RPL010), **LEB** (DMT070, TPL033, RPL205), **Chromocytogenes Agar** (DMT700+SMT700, PPL970), **Alk-Saline Broth** (DMT159), **Vibrio Hipersaline Broth** (DMT137, TPL502), **TCBS Agar** (DMT119, TPL506, PPL922), **COMPACT-DRY-PLATES®-VP** (1000900), **TSC Agar** (DMT175, TPL137), **Baird Parker Agar** (BCD085+SBH011, TPL125+SBH011, RPL003+SBH011), **COMPACT-DRY-PLATES®-SA** (1000899), **Rosa Bengala Caf.Agar** (DMT101, TPL072, RPL034), **Sabouraud Caf. Agar** (DMT102, TPL073, RPL035), **COMPACT-DRY-PLATES®-YM** (1000869), **PCA** (BCD010, TPL071, RPL030), **CHROMOGENIC PCA** (BCD510, TPL271, RPL530), **COMPACT-DRY-PLATES®-TC** (1000166), **VRBG Agar** (BCD088, TPL058, RPL047), **COMPACT-DRY-PLATES®-ETB** (1002941),

meet the VALIDATION standards of UNE-EN-ISO 16140:2003, the results of which are attached. The validation has been performed by comparison, using quantitative certified and traceable strains versus official benchmark methods (Various Technical Standards in use or in ISO/UNE final approval stage for food microbiology).

The present certificate is only valid for the period of validity of the cited methods and although it is guaranteed every three months by SEILA comparative revalidation, it must be renewed within five years from the date of issue indicated below.

This certificate authorises the user of the method and validated medium to use the MICROKIT validation studies to endorse the internal validation of their method with their own matrices, teams, analysts and facilities, providing that they correctly use the methods and products referenced in and covered by this certificate.

Guaranteed by:

Date: 16-07-2009

Jorge Sanchis Solera
SEILA Coordinator and MICROKIT® Quality Manager

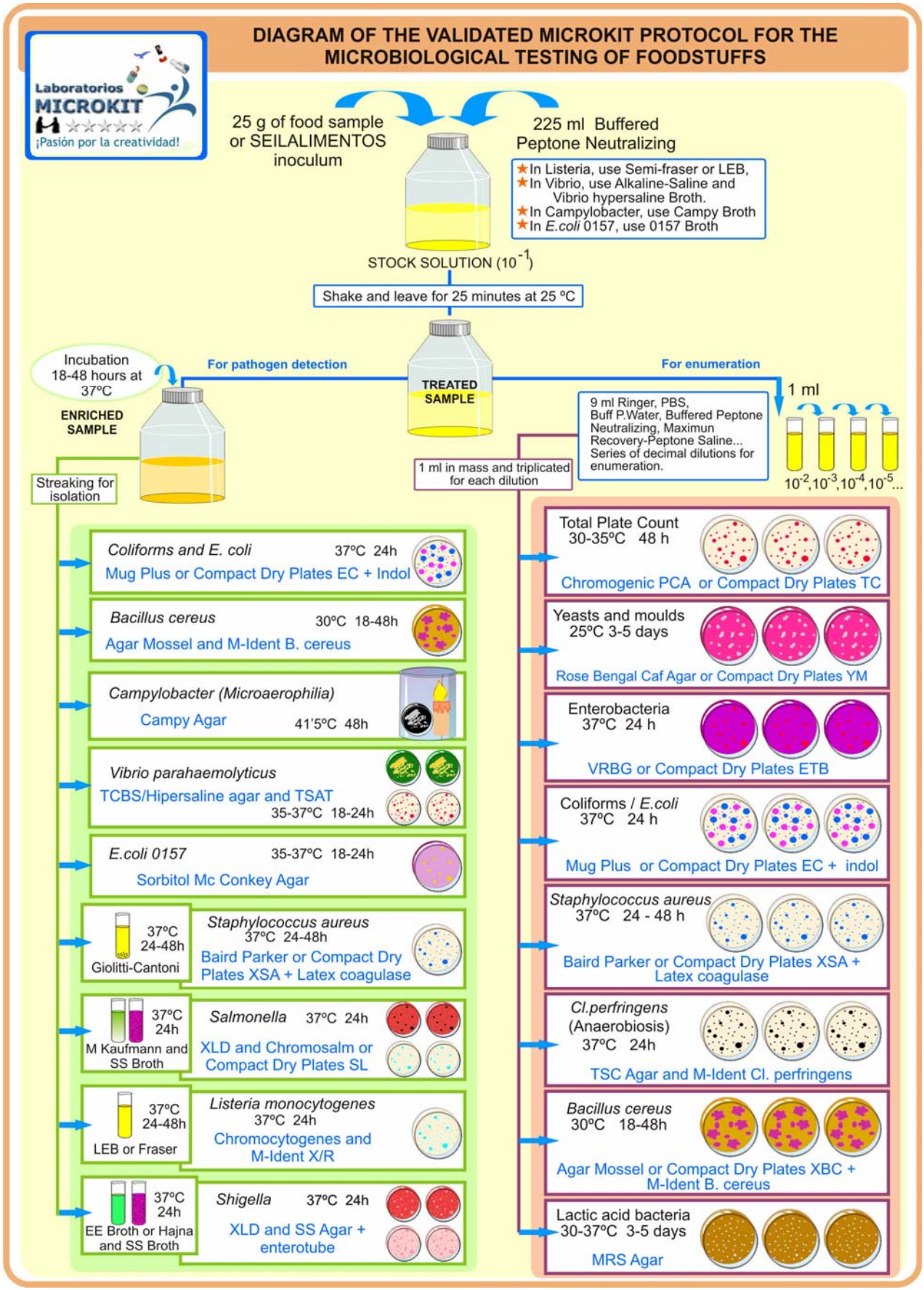


DIAGRAM OF THE VALIDATED MICROKIT PROTOCOL FOR THE MICROBIOLOGICAL TESTING OF FOODSTUFFS
Laboratorios MICROKIT ¡Pasión por la creatividad!
25 g of food sample or SEILALIMENTOS inoculum
225 ml Buffered Peptone Neutralizing
In Listeria, use Semi-fraser or LEB,
In Vibrio, use Alkaline-Saline and Vibrio hypersaline Broth.
In Campylobacter, use Campy Broth
In E.coli 0157, use 0157 Broth
STOCK SOLUTION (10-1)
Shake and leave for 25 minutes at 25 ºC
TREATED SAMPLE
For pathogen detection
For enumeration
Incubation 18-48 hours at 37ºC
ENRICHED SAMPLE
Streaking for isolation
1 ml in mass and triplicated for each dilution
9 ml Ringer, PBS, Buff P.Water, Buffered Peptone Neutralizing, Maximun Recovery-Peptone Saline... Series of decimal dilutions for enumeration.
1 ml
10-2,10-3,10-4,10-5...
Coliforms and E. coli 37ºC 24h
Mug Plus or Compact Dry Plates EC + Indol
Bacillus cereus 30ºC 18-48h
Agar Mossel and M-Ident B. cereus
Campylobacter (Microaerophilia)
Campy Agar 41'5ºC 48h
Vibrio parahaemolyticus
TCBS/Hipersaline agar and TSAT 35-37ºC 18-24h
E.coli 0157 35-37ºC 18-24h
Sorbitol Mc Conkey Agar
37ºC 24-48h Giolitti-Cantoni
Staphylococcus aureus 37ºC 24-48h
Baird Parker or Compact Dry Plates XSA + Latex coagulase
37ºC 24h M Kaufmann and SS Broth
Salmonella 37ºC 24h
XLD and Chromosalm or Compact Dry Plates SL
37ºC 24-48h LEB or Fraser
Listeria monocytogenes 37ºC 24h
Chromocytogenes and M-Ident X/R
37ºC 24h EE Broth or Hajna and SS Broth
Shigella 37ºC 24h
XLD and SS Agar + enterotube
Total Plate Count 30-35ºC 48 h
Chromogenic PCA or Compact Dry Plates TC
Yeasts and moulds 25ºC 3-5 days
Rose Bengal Caf Agar or Compact Dry Plates YM
Enterobacteria 37ºC 24 h
VRBG or Compact Dry Plates ETB
Coliforms / E.coli 37ºC 24 h
Mug Plus or Compact Dry Plates EC + indol
Staphylococcus aureus 37ºC 24 - 48 h
Baird Parker or Compact Dry Plates XSA + Latex coagulase
Cl.perfringens (Anaerobiosis) 37ºC 24h
TSC Agar and M-Ident Cl. perfringens
Bacillus cereus 30ºC 18-48h
Agar Mossel or Compact Dry Plates XBC + M-Ident B. cereus
Lactic acid bacteria 30-37ºC 3-5 days
MRS Agar

❖ VALIDATION METHOD

For each microbiological parameter involved, using a minimum of 20 samples, the presence/absence and the enumeration obtained using the MICROKIT method (PRT-SEILA-001 and PRT-AL-001 to PRT-AG-017 foodstuff protocol) was compared to quantitative HPA certified strains using the official method (current ISO/UNE Technical Standards for food microbiology). T MICROKIT culture media described in the quoted protocols, and described here, were used.

It is to be noted that the various CompactDryPlates® have already been validated by AOAC, Microval and NordVal. For this reason, although we have not included many samples, due to their novelty in Spain, the data of these validations is confirmed. Our intention was to widen the range of food matrices and verify that they function correctly in all of them, as was the case (see the bibliography at the end of this certificate).

MICROKIT® Chromosalm for Salmonella has also been validated previously, in this case in a joint project involving 6 laboratories and 250 samples compared from all types of natural matrices. This was published in the XIX Microbiology Congress in Santiago de Compostela and in May 2003 in the review “Técnicas de Laboratorio”. Its excellence is once again confirmed in this latest validation study. In addition, MICROKIT ® Chromocytogenes is exactly the Ottaviani & Agosti Agar of ISO 11290:2004, but we wanted to check (as it is not always the case) that the formula expressed therein is correct, as is patently clear from the results of this report. Furthermore, the MICROKIT® Rosa Bengala Caf.Agar has been validated by the public for years, since we published its comparative excellence in 1996, so that we need only confirm that its commercial success is due to the superb quality of all its validation parameters. Finally, the MICROKIT® MugPlus Agar has also been validated with excellence and its equivalence has been proven in aquatic matrices up to the point of its formula being recommended in the Decree SCO/778/2009 of the Ministry of Health and Consumer Affairs (17 March, published in the BOE Official Journal of 31 March 2009.

In addition, all data was compared with results obtained in 42 identical types of food matrices with identical inocula by 100 Spanish laboratories participating in the SEILAPALIMENTOS comparative service over the last 10 years, with special emphasis on the most recent years, from 2007-2009, with a total of 42 batches, resulting in a comparison of over **730 identical samples** between participants that use the BENCHMARK METHOD and the MICROKIT® method, which is used for its periodical revalidation. Given the consistency of the results, summarised in a comparative study "June/2008: Conclusions regarding the validation of MICROKIT® protocols optimized for the **microbiological analysis of foodstuffs** versus the relevant standards, by means of Seilalimentos comparative tests. Técnicas de Laboratorio 341, 5/2009. XVI National Congress of food microbiology. Córdoba, 9/2008". In this most recent validation, not all sample data is taken into account, but rather that of the last three years (12 intercomparative circuits between 2007 and 2009), since doing so would only increase statistical work which would finally be redundant. Furthermore, in these studies of the last three years, the inocula are of quantitative certified strains, so that we obtained two simultaneous methods of contrast: standard strains and the paired comparison method. Of the 100 participating laboratories, at least 6 were accredited for microbiological analysis by ISO 17025, as required by ISO 17994 for establishing equivalence between microbiological methods.

Restrictions of use of the MICROKIT® protocol, processes and kits: the scope of the validation includes all types of food matrices, as the MATRICES used included cinnamon, 3 flavours of ice cream, cakes, mortadella, cheese, raw mussels, chicken, pre-cooked rice with green beans, raw hamburgers, chocolate and hazelnut cream, mayonnaise, orange juice, raw fish, pasteurised milk, Frankfurt sausages, paprika, raw egg, salt, powdered milk, malt, fish meal, glucose, powdered meat cubes, lactose, soya, yeast autolysate, orange extract, tiger nut milk, biscuits, pasta, extruded cereals, mashed potatoes, animal feed, milk, jars of baby food and cocoa.

❖ RESULTS

In blue, the MICROKIT results for the detection and enumeration in any presentation.
In black, the results of the benchmark method. Third methods are not included, although other much-used methods are indicated, nor are SEILALIMENTOS participants that do not strictly apply the benchmark method.

1. RESULTS OF QUALITATIVE PARAMETERS

PARAMETER	SENSITIVITY (S) (scarcity of False Negatives) (F-)		SPECIFICITY (SP) (scarcity of False Positives) (F+)	
	% MICROKIT® METHOD	% BENCHMARK METHOD	% MICROKIT® METHOD	% BENCHMARK METHOD
Salmonella spp.	Broth SS MICROKIT®: 7 F- / 40: **82.5 %** S (1) - - - - - - - - - - - - - - - - - - Chromosalm MICROKIT®: 6 F- / 75: **92.0 %** S (1) - - - - - - - - - - - - - - - - - - CompactDryPlates®-SL 0 F- / 18: **100%** S	ISO 6579 Salmonella: 28 F- / 74: **62.2 %** S	Broth SS MICROKIT®: 0 F+ / 5: **100 %** SP - - - - - - - - - - - - - - - - - - Chromosalm MICROKIT®: 0 F+ / 7: **100 %** SP - - - - - - - - - - - - - - - - - - CompactDryPlates®-SL 0 F+ / 6: **100 %** SP	ISO 6579 Salmonella: 3 F+ / 16: **81.2 %** SP
Shigella spp.	Broth SS MICROKIT®: 6 F- / 36: **83.3 %** S (1) - - - - - - - - - - - - - - - - - - Chromosalm MICROKIT®: 2 F- / 30: **93.3 %** S (1) - - - - - - - - - - - - - - - - - - CompactDryPlates®-SL 0 F- / 8: **100%** S	ISO 21567 Shigella: 17 F- / 21: **19.0 %** S	Broth SS MICROKIT®: 0 F+ / 39: **100 %** SP - - - - - - - - - - - - - - - - - - Chromosalm MICROKIT®: 0 F+ / 39: **100 %** SP - - - - - - - - - - - - - - - - - - CompactDryPlates®-SL 0 F+ / 6: **100%** S	ISO 21567 Shigella: 1 F+ / 26: **96.2 %** SP
Bacillus cereus	JOINT METHOD:ISO 7932 Agar Mossel PREP-MYP 5 F- / 36: **86.1 %** S		JOINT METHOD ISO 7932 Agar Mossel PREP-MYP 2 F+ / 17: **88.2 %** E	
E.coli O157	CompactDryPlates®-EC 0 F- / 5: **100 %** S	ISO 16654 Sorb.McConkey 2 F- /. 19: **89.5 %** S	CompactDryPlates®-EC 0 F+ / 3: **100 %** SP	ISO 16654 Sorb.McConkey 0 F+ / 12: **100 %** SP
E.coli total (3)	MUGPLUS MICROKIT®: 4 F- / 28: **85.7 %** S (1) - - - - - - - - - - - - - - - - - - CompactDryPlates®-EC 0 F- / 16: **100%** S	ISO 7251 BGBL 2% Broth 3 F- / 11: **72.7 %** S - - - - - - - - - - - - - - - - - - ISO 16649 TBX Agar 2 F- / 19: **89.5 %** S	MUGPLUS MICROKIT®: 0 F+ / 24: **100 %** SP - - - - - - - - - - - - - - - - - - CompactDryPlates®-EC 0 F+ / 12: **100%** SP	ISO 7251 BGBL 2% Broth 1 F+ / 13: **92.3 %** SP - - - - - - - - - - - - - - - - - - ISO 16649 TBX Agar 0 F+ / 12: **100 %** SP
Pseudomonas aeruginosa	JOINT METHOD: CETRIMIDE AGAR 2 F- / 27: **92.6 %** S		JOINT METHOD: CETRIMIDE AGAR 3 F+ / 43: **93 %** E	
Listeria monocytogenes	ISO 11290 modern- Chromocytogenes MICROKIT®: 4 F- / 68: **94.1 %** S (1)	ISO 11290 standard- Palcam/Oxford 9 F- / 35: **74.3 %** S	ISO 11290 modern- Chromocytogenes MICROKIT®: 1 F+ / 14: **92.9 %** SP (2)	ISO 11290 standard- Palcam/Oxford 0 F+ / 12: **100 %** SP
Clostridium perfringens or sulfite-reducing clostridia	JOINT METHODS: ISO 7937 TSC Agar 8 F- de 13: **38,5 %** S TSN: 1 F- de 2: **50 %** S SPS: 1 F- de 1: **0 %** S		JOINT METHODS: ISO 7937 TSC Agar 3 F+ / 76: **96 %** SP TSN: 2 F+ / 25 **92 %** SP SPS: 0 F+ / 9: 10**0 %** SP	
Staphylococcus aureus (4)	CompactDryPlates®-XSA 0 F- / 6: **100 %** S	ISO 6888 BAIRD-PARKER 29 F- / 91: **68.1 %** S - - - - - - - - - - - - - - - - - - ISO 6888 RPF 8 F- / 21: **61.9 %** S	CompactDryPlates®-XSA 0 F+ / 14: **100 %** SP	ISO 6888 BAIRD-PARKER 17 F+ / 78: **78.2 %** SP - - - - - - - - - - - - - - - - - - ISO 6888 RPF 11 F+ / 42: **73.8 %** SP
Fungi (yeasts and moulds) (2)	Rosa Bengala Caf. MICROKIT®: 7 F- / 45: **84.4 %** S (1) - - - - - - - - - - - - - - - - - - CompactDryPlates®-YM 3 F- / 31: **90 %** S (1)	UNE 34821 Sabouraud Caf. 11 F- / 39: **71.8 %** S - - - - - - - - - - - - - - - - - - ISO 7954 YGC (Caf.Glucose) 5 F- / 23: **78.3 %** S - - - - - - - - - - - - - - - - - - CENAN OGYE Agar 1 F- / 9: **88.9 %** S	Rosa Bengala Caf. MICROKIT®: 0 F+ / 3: **100 % SP** - - - - - - - - - - - - - - - - - - CompactDryPlates®-YM 3 F+ / 10: **70 %** SP	UNE 34821 Sabouraud Caf. 0 F+ / 6: **100 %** SP - - - - - - - - - - - - - - - - - - ISO 7954 YGC (Caf.Glucose) 1 F+ / 6: **83,3 %** SP - - - - - - - - - - - - - - - - - - CENAN OGYE Agar 0 F+ / 4: **100 %** SP
Total aerobics (2)	PCA-CROMOGÉNICO MICROKIT®: 0 F- / 15: **100 %** S - - - - - - - - - - - - - - - - - - CompactDryPlates®-TC 0 F- / 44: **100 %** S	ISO 4833 PCA 7 F- / 105: **93.3 %** S	N/A	
Enterobacteria (2)	CompactDryPlates®-ETB 4 F- / 30: **86.7 %** S	ISO 21528 VRBG 14 F- / 126: **88.9 %** S	N/A	
Coliforms (2, 3)	MUGPLUS MICROKIT®: 4 F- / 22: **81.8 %** S (1) - - - - - - - - - - - - - - - - - - CompactDryPlates®-EC 1 F- / 31: **96.8 %** S	ISO 7251 BGBL 2% Broth 5 F- / 20: **75 %** S - - - - - - - - - - - - - - - - - - VRBL: 5 F- / 40: **87.5%** S	MUGPLUS MICROKIT®: 0 F+ / 6: **100 %** SP - - - - - - - - - - - - - - - - - - CompactDryPlates®-EC 0 F+ /12: **100 %** SP	ISO 7251 BGBL 2% Broth 0 F+ / 9: **100 %** SP - - - - - - - - - - - - - - - - - - VRBL: 1 F- / 9: **88.9%** S

(1). Sensitivities of under 95%, but higher than that of the benchmark reference, prove the suitability of use of the MICROKIT® method for *Salmonella spp., Shigella spp., E.coli, Listeria monocytogenes*, Fungi (yeasts and moulds) and Coliforms.

(2). Although these are eminently quantitative parameters, non-detection is generally a result of mass seeding with over-warm agar or surface seeding with inocula that is insufficiently shaken. Working with methods that minimise these two critical points (Compact-Dry-Plates®, in the first case and forceful shaking immediately after seeding in the second case), laboratories will increase their efficiency. This is especially important in the case of fungi, where false negatives are generally due to the absence of shaking immediately before each dilution and seeding, rather than to the culture medium, as the mould spores float in a matter of seconds.

(3). The Biomerieux Coli ID medium has been included in this validation, due to the frequency with which it is used in food microbiology in our country, which is higher even than that of MugPlus. Validation data shows that, despite being a better medium than the benchmark, MugPlus outperforms it in all quality parameters. For *E.coli*, MICROKIT® MUGPLUS obtains 85.7 **%** Sensitivity and Coli ID, 73% and they both have 100% specificity. For Coliforms, MICROKIT® MUGPLUS obtains 81.8 % Sensitivity and Coli ID, 73.7%; and as regards specificity, MICROKIT® MUGPLUS obtains 100 % and Coli ID, 83.3 %.

(4). There is an increasingly-used chromogenic medium for *Staphylococcus aureus* which, due to its detrimental sensitivity in food samples (0%), we have to declare invalid. It probably comes from the market for clinical samples, which is totally different to the food sector, as the first are generally massive pure cultures whilst the second are completely the contrary, with a poor concentration of the target microorganisms and a high interferant load.

Data from the previous 1999 - 2006 study from the bibliography:

PARAMETER	DATA 1999 TO 2006 EFFICIENCY: SENSITIVITY + SPECIFICITY (scarcity of False Negatives and False Positives)	
	% MICROKIT® METHOD	% BENCHMARK METHOD
Salmonella spp.	MICROKIT® SS Broth: 100 % EFFICIENCY MICROKIT® Chromosalm Agar: 96.4 % EFFICIENCY	ISO 6579, ISO 6579: 77 % EFFICIENCY
Shigella spp.	MICROKIT® SS Broth: 100 % EFFICIENCY	ISO 21567: 77 % EFFICIENCY
Bacillus cereus	IS THE SAME METHOD:	ISO 7932: 75 % EFFICIENCY
E.coli O157	WAS THE SAME METHOD:	ISO 16654: 90 % EFFICIENCY
E.coli total	MICROKIT® MUGPLUS: 94.5 % EFFICIENCY	ISO 7251, ISO 16649: 77 % EFFICIENCY
Pseudomonas aeruginosa	IS THE SAME METHOD:	UNE-EN-ISO 12780: 82 % EFFICIENCY
Listeria monocytogenes	WAS THE SAME METHOD:	ISO 11290: 66 % EFFICIENCY
Clostridium perfringens or sulfite-reducing clostridia	IS THE SAME METHOD:	UNE EN 13401: 72 % EFFICIENCY
Staphylococcus aureus	WAS THE SAME METHOD:	ISO 6888: 65 % EFFICIENCY
Fungi (Yeasts and moulds)	MICROKIT® Rosa Bengala Caf.: 100% EFFICIENCY	ISO 7954, CENAN, UNE 34821, 70 % EFFICIENCY

It can be noted that the SEILALIMENTOS comparative records of previous years (1999-2006) lead to the same conclusions as the current SEILALIMENTOS 2007-2009 validation with quantitative strains and comparison. In all cases, the MICROKIT® method is more efficient than the traditional method, especially as regards its far higher Sensitivity in all parameters. This is not surprising, since all of the methods were created as a result of the unsatisfactory results obtained

with standard methods and when they are redesigned to conform, their improved efficiency will be verified.

The detection limits are also remarkably optimized using the MICROKIT® protocols, although initially this was not obviously the case. The data shows numerous cases of detection with our methods when the inocula is low, that the benchmark method was unable to detect either in the pilot laboratory or in those of the other participants of the comparative study. We do not have data regarding what would happen with inocula that is between the minimum currently detected with MICROKIT® methods and the minimum currently detected with the benchmark methods in this study, since we do not have inocula values of, for example 25 ufc of *E.coli* O157. Logic suggests that the differences between the two methods would not be so wide. However, we can assert, with the data we have available, that the MICROKIT® method proves the accuracy of the protocol in detecting target microorganisms, even when they are found in very low concentrations and in the presence of numerous interferants or their associated interferants.

PARAMETER	**DETECTION LIMIT CONFIRMED AS FROM*:**	
	MICROKIT METHOD	BENCHMARK METHOD
Salmonella spp.	3 ± 1 ufc/25 g	4-16 ufc/25 g
Shigella spp.	80 ± 10 ufc/25 g	>>>171 ufc/25 g
Bacillus cereus	129 ± 9 ufc/g	
E.coli O157	15 ± 3 ufc/25 g	80 ± 10 ufc/25 g
E.coli total	92 ± 7 ufc/g	>>>240 ufc/g
Pseudomonas aeruginosa	95 ± 7 ufc/g	
Listeria monocytogenes	29 ± 4 ufc/g	29 ± 4 ufc/g
Clostridium perfringens or sulphite-reducing clostridia	50-70 ufc/g	
Staphylococcus aureus	202 ± 12 ufc/g	>>> 480 ufc/g
Fungi (Yeasts and moulds)	19 ± 3 ufc/g	30 ± 4 ufc/g
Enterobacteria	2-20 ufc/g	20 ± 3 ufc/g
Coliforms	20 ± 3 ufc/g	20-92 ufc/g

* It should be noted that microbiological uncertainty, due to the contagious distribution of microorganisms (Poisson or negative binomial), prevents us from asserting that two sub-samples from any recently-shaken foodstuffs are identical. This therefore prevents us from maintaining, in a statistically reliable manner, detection limits lower than those given, since there may be 1 ufc in one sub-sample, 2 in another and none in yet another. It is noted that in all cases, the MICROKIT® method comes much closer to the ideal detection limit (theoretically 1 ufc/x g) than the benchmark reference, in a statistically highly significant manner. The imprecision resulting from the impossibility of improving the consistent distribution of strains in the sub-samples enables us to presume that the detection limit is actually much closer to the ideal of 1 ufc/x g in 100% of the samples using the methods optimized by MICROKIT®.

2. RESULTS OF QUANTITATIVE PARAMETERS

We include the quantitative data of all the parameters when available, including for those where only investigation/detection is required, in order to obtain the maximum amount of data as regards the relative efficacy of each method:

PARAMETER	ACCURACY (measured in relative recovery: proximity of the counts to the standard value-benchmark inocula value or accepted value in the comparison)		PRECISION (dispersion of results measured in repetition and reproducibility, capacity to give equivalent results) Although this depends more on factors other than the process/ method, we have included it for the sake of purity)	
	% MICROKIT® METHOD	% BENCHMARK METHOD	% MICROKIT® METHOD	% BENCHMARK METHOD
Total aerobics	Compact-Dry-Plates® TC: 141 % *** - - - - - - - - - - - - - - - - - - Chromogenic PCA: 91 % *	PCA: 67% *	Compact-Dry-Plates® TC: ± 0.44 log and 4% of samples out of range: OK - - - - - - - - - - - - - - - - - - Chromogenic PCA:: ± 0.07 and 14% out of range : OK	PCA ± 0.48 log and 4% of samples out of range
Yeasts and moulds	Rosa Bengala Caf.Agar 189 % *** - - - - - - - - - - - - - - - - - Compact-Dry-Plates® YM 81.7 % *	Sabouraud Caf.Agar 131%*** YGC 212 % *** OGYE 208 % ***	Rosa Bengala Caf.Agar < ± 0.03 log, (and 10 % of samples out of range): OK	Sabouraud Caf.Agar ± 0.29 log and 33% of samples out of range / YGC ± 0.19 log and 8% out of range / OGYE ± 0.17 log and 25% out of range
Enterobacteria	Compact-Dry-Plates® ETB 127 % ***	VRBG: 73 % *	Compact-Dry-Plates® ETB ± 0.07 log and 0 % out of range OK	VRBG ± 0.01 log and 2.5 % of samples out of range
Coliforms	Compact-Dry-Plates® EC: 597 % *** MUGPLUS: 175 % ***	VRBA: 31 % * BGBL: 95 %*	Compact-Dry-Plates® EC: < ± 0.04 log, (and 0 % of samples out of range): OK MUGPLUS< ± 0.5 log and 0 % out of range): OK	VRBA: ± 1.05 ** and 0% of samples out of range BGBL:± 0.02 and 0% of samples out of range
E.coli	Compact-Dry-Plates® EC: 356 % *** MUGPLUS: 117 % ***	TBX: 36 % * BGBL: 26.5 %*	Compact-Dry-Plates® EC: *< ± 0.07 (and 9 % of samples out of range):* OK MUGPLUS ± 0.4 log and 0% of samples out of range: OK	TBX ± 0.07 log and 0% samples out of range BGBL:± 1.45 ** and 0% of samples out of range
Bacillus cereus	AGAR MOSSEL PREP-MYP 379 %*		Not enough data	
Listeria monocytogenes	CHROMOCYTOGENES: 113% ***	PALCAM: 148%***	Not enough data	
Staphylococcus aureus	Compact-Dry-Plates® XSA: 80% *	Baird Parker Agar 95 %* RPF: 121 % ***	Compact-Dry-Plates® STAF: < ± 0.1 log and 14% of samples out of range: OK	Baird Parker Agar < ± 0.06 and 9 % of samples out of range RPF: < ± 0.02 log and 6 % of samples out of range

* All this is fully within the standard statistical tolerance value of ± 2 log, acceptable. Even so, VRBA methods for coliforms, together with TBX and BGBL for *E.coli*, using the relative recovery method, do not prove to be as precise as should be expected from the benchmark method. The standard PCA is much less precise than the chromogenic or the Compact-Dry-Plates® TC, because both these methods avoid confusing colonies with particles. The VRBG is much less precise than the Compact-Dry-Plates®-ETB because mass seeding in warm agar renders many targets microorganisms inviable.

** Although all precisions are within the statistically acceptable ± 2 log, the poorest are those obtained by VRBA for coliforms and BGBL for *E.coli*

*** These work even better than the standard strains, as their enumeration refers to other standard methods/medium, such as Agar-Sangre or EMB Levine.

Also noteworthy is the maximum precision of Compact-Dry-Plates®-EC and ETB, together with the fact that 0% of aberrant samples were included that would have been rejected from the study for being out of range.

We wish to emphasize, as regards precision, that a considerable part of the imprecision detected may be due to the analyst component and the inter-laboratory component, rather than to the culture medium component or its format.

❖ CONCLUSIONS

1-It can be noted that all the methods proposed by MICROKIT® in its protocols equal or even IMPROVE the analytical results of the benchmark methods:

a) The various chromogenic culture media (MugPlus, Chromosalm, Chromocytogenes, Chromogenic PCA), together with Salmonella-Shigella Broth and Rosa Bengala Caf. Agar, prove to be more efficient than their standard counterparts. They all show higher sensitivity, specificity, detection limits, accuracy and/or precision than the standard benchmark methods, as a result of years, almost decades, of collaborative and comparative work, guaranteeing the maximum efficiency of all of them. These MICROKIT® media prove the effectiveness of its protocol in detecting targets microorganisms, even when found in very low concentration and in the presence of numerous interferants or their associated interferants, saving the laboratory from unnecessary confirmations of unsought microorganisms. For all these reasons, they are validated.

b) The Compact-Dry-Plates® maximise sensitivity, specificity, accuracy and precision, saving the critical point of the mass seeding for remelting agars which, in the standard method, often render the target microorganisms inviable due to excessive heat. All the Compact-Dry-Plates® described in this study are validated and their validation data proves that they are not only the most convenient and practical method of working in microbiology, but also the most reliable.

2-All the MICROKIT® methods described here, when followed to the letter with our medium and kits, and independent of their presentations, are validated, as they are at least as reliable as the benchmark method with which they were compared and, for the majority, even more so. Although some do not exceed 95% efficiency as regards the inocula value, they are all more efficient than the benchmark methods. They detect and/or count, in all cases, sufficient concentrations of all the groups of microorganisms studied: *Salmonella spp., Shigella spp., Bacillus cereus, E.coli O157, E.coli, Pseudomonas aeruginosa, Listeria monocytogenes, Clostridium perfringens, Staphylococcus aureus, Fungi –yeasts and moulds-,* Total aerobics, Enterobacteria and Coliforms. In addition, they do so at least as well as the benchmark reference, demonstrating far higher sensitivity and specificity, closer proximity to the minimum necessary detection limit, as well as better robustness, ease of use and economy than the benchmark method, when implemented in the various laboratories that apply them.

3-The scarcity of false positives, or improved specificity, enables laboratories to save time and money as regards confirmations of false positives that are necessary with the benchmark method. The time saved could be used for performing more analyses, so the implementation of these optimized techniques increases the overall yield of the laboratory.

4-The scarcity of false negatives, or maximum sensitivity, makes use of these methods recommendable. The improved ease of use of the MICROKIT® methods enables more samples to be treated in less time, while reducing the number of critical points of the analysis, thus increasing its robustness.

5-The use of Salmonella-Shigella Broth as a substitute for Rappaport Broth increases sensitivity of Salmonella detection by 20% as regards compliance with ISO 6579 for Salmonella. This means that up to 20% of samples with these pathogens may be undetected with the current official method. In one of the most searched for and dangerous food transmission microorganisms, it is worth such a simple change in medium in order to increase detection in all types of laboratories.

6-The use of Cromosalm Agar as a second isolation agar in ISO 6579 increases the sensitivity of *Salmonella spp.* detection by over 30% in complex samples compared to the use of other standard medium (BGA, SS Agar, Hektoen, etc.) and even other modern chromogenics

(Magenta-Gal). This means that up to 30 % of samples with this pathogen may be undetected with the current official method.

7-The use of MugPlus Agar proves to be the best choice for detecting and counting Coliforms and *E.coli*, as it combines maximum sensitivity, specificity, accuracy and precision. Another medium with considerable commercial success (Coli-ID from Biomerieux) also performs well, but not as well as MugPlus. However, the relative recovery method shows that TBX and BGBL for *E.coli* and VRBA for coliforms do not perform as well as regards accuracy as would be expected from the benchmark method. In addition, VRBA for coliforms and BGBL for *E.coli* are very imprecise.

8-Application of the 2004 addendum to ISO 11290 of *Listeria monocytogenes* enables laboratories to increase sensitivity by 20%, thanks to the Ottaviani & Agosti medium, that we call Chromocytogenes in MICROKIT®. We urge all laboratories to shelve the Oxford and Palcam and substitute instead this excellent medium of Italian design.

9-The low sensitivity of all sulfite-reducing clostridia and *Cl.perfringens* detection and enumeration methods, although not as alarming as in water, shows that the methods are not highly refined. These microorganisms, strict anaerobics, grow with white or grey colonies that give false negatives as they are not black, when this is actually their growth form when the anaerobiosis is not strict and cannot properly reduce the sulphites. Ferric ammonium citrate and sodium metabisulfate are thermolabile, which gives them such poor results in this parameter. They should be added aseptically, as a sterile supplement (MICROKIT® VMT136), after autoclaving and cooling any of the medium involved, although already incorporated (SPS, TSC, TSN, Sulfite Iron Agar, Lactose Sulfite Broth, etc.).

10-The interpretation of coagulase in Baird Parker and RPF is highly controversial and one of the greatest sources of analytical error in foodstuffs. Both medium are invalid for waters and cosmetics and although validation data in foodstuffs is acceptable, it is still the most controversial parameter in food microbiology. For this reason, CompactDryPlates®-XSA, which have a completely different basis and took 3 years to design, should be taken more into account.

11-Methods for enumerating fungi (yeasts and moulds) are surprisingly good in all cases (YGC-Caf.Glucose Agar, OGYE, Sabouraud Caf., Rosa Bengala Caf.Agar), but our preference for the Rosa Bengala Caf.Agar is due to the fact that, in addition, the invasive moulds do not expand in this medium, enabling them to be counted in all plates and with it having been demonstrated in our previous study (*Comparación entre los diversos medios comerciales para aislamiento de hongos -levaduras y mohos-: Excelencia del Rosa Bengala Caf.Agar. Técnicas de Laboratorio, Nº 211. 05/1996*) that the diversity of yeasts and moulds that a same plate recovers is maximized in this medium. Its unmistakable colour saves having to mark the name on the plates.
In addition, we note that many laboratories obtain exceedingly low results (not taken into account in the validation, due to aberration) independently of the medium used. This is due to the mould spores floating in a matter of seconds, meaning that if they are not shaken immediately before each dilution and each seeding, they are lost on the surface of the Broths, giving rise to false negatives or very low recovery.

12-Substituting standard PCA with chromogenic PCA enables not only the colonies of particles to be better distinguished, but also, and especially, to see small colonies that are not detected in PCA. This is not a mere anecdote, since the difference in validated relative recovery between chromogenic PCA and standard PCA is no less than 24% ufc. In other words, chromogenic PCA recovers an average of 124% compared to standard or, put differently, standard PCA recovers only 81% of the aerobic flora that chromogenic PCA is capable of detecting. As regards CompactDryPlates®-TC, relative recovery is over twice that of standard PCA. In other words, it recovers less than 50% of the aerobic flora present, which is not a good result for a benchmark method. Two critical points are combined in standard PCA: the confusion of colonies with food particles, and mass seeding in warm agar that renders inviable a good many of the aerobics present.

13- The relative recovery method for Enterobacteria shows that VRBG is not as good as should be expected from the benchmark method, as it recovers 73% of the inocula value and only 57.5% as regards enumeration of enterobacteria actually present that can be detected by the CompactDryPlates®-ETB method. As with PCA, these very poor VRBG results point to the thermal shock suffered when mass seeding in warm agar, a critical point that does not occur with the method recommended by MICROKIT®.

14-Some laboratories commit serious lack of detection errors because they do not use appropriate medium. For example, Sorbitol MacConkey cannot be used for *E.coli* (when it is a differential medium, only intended for the detection of its enterohaemorrhagic 0157 strains), nor can VRBG be used for coliforms (when it is a glucose medium, for Enterobacteria), nor VRBL for Enterobacteria (when it is a lactose medium, for coliforms). Saving the cost of a culture medium may have serious consequences for public health due to the issue of analysis certificates that are so irresponsibly signed. There are also laboratories that use media designed for clinical microbiology, without them having been previously validated for food microbiology, as is indicated by 0% sensitivity results for a chromogenic agar for *Staphylococcus aureus* with (even so) increasing commercial success.

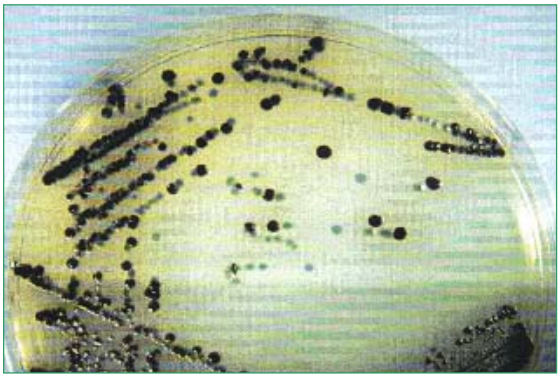
15-Laboratories should not be surprised if they obtain counts that are systematically higher than those certified in the quantitative strains they use, when they use medium and kits of outstanding quality, since those certified refer to other medium of other brands (however good they are) and with other characteristics.

16- We are confident that all this work – the fruit of a tremendous joint effort over the last 7 years by 100 of the many laboratories all over Spain that use some of the MICROKIT® methods, medium and/or kits, will be well-accepted by the accrediting, standards and inspection bodies. All this is in order to improve analytical quality and for lowering cost and waste for laboratories and the environment involved with these methods. This would provide incentives that, from the viewpoint of this company, would enable us to continue designing more efficient methods in Spain for other microbiological parameters and save ISO 17025 accredited laboratories, as well as those authorised by the Ministry of Health, from stagnating with standard methods that we have proven to be highly optimizable, thanks to a concept that should never be forgotten in any laboratory: continuous improvement without bureaucratic restrictions to limit it.

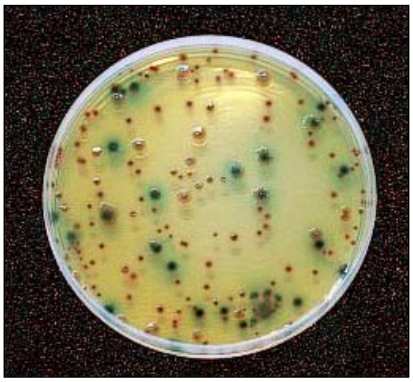
17- We also hope that laboratories will take advantage of all these conclusions to implement, if they are not already used, the most efficient methods. Not only will they obtain more reliable results, but they will also save time, through more advanced and selective techniques, and money by avoiding many of the confirmations which would no longer be necessary. We would like to remind laboratories that these validation conclusions apply exclusively to the described media and kits of the MICROKIT® brand, as using other brands would require total revalidation because it can never be ascertained that they will behave in the same manner. MICROKIT ® and Compact-Dry-Plates ® are registered trade names, owned by Laboratorios MICROKIT, S.L. Any suppliers offering these products that are not official Laboratorios MICROKIT, S.L. dealers are committing an offence of misappropriation and the illegal use of trade names. The owner of the trade name should be immediately informed by the laboratory that detects the offence. It is easy to understand that we do not work for our competitors!

❖ PHOTOGRAPHIC APPENDIX

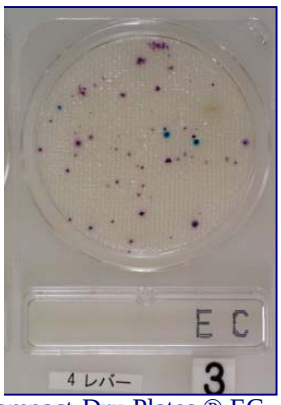
SS Broth MICROKIT®



Chromosalm Agar MICROKIT®



MUGPLUS AGAR

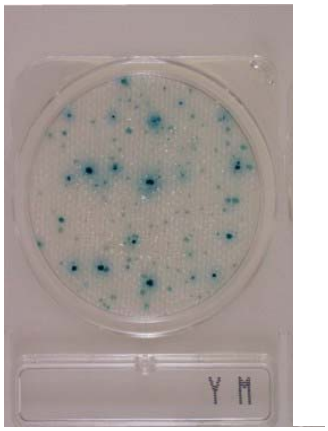


Compact-Dry-Plates ® EC

Chromocytogenes Agar

Compact-Dry-Plates®-XSA

Rose Bengal Caf.Agar



Compact-Dry-Plates®-YM

Cromogenic-PCA MICROKIT®

Compact-Dry-Plates ® TC

Compact-Dry-Plates®-ETB

❖ BIBLIOGRAPHY

- 1992. MINISTERIO DE SANIDAD Y CONSUMO: Microbiologia alimentaria. Metodología analítica para alimentos y bebidas. Díaz de Santos.
- 1982. MINISTERIO DE SANIDAD Y CONSUMO: Técnicas para el análisis micriobiol´ñogico de alimentos y bebidas del CENAN Instituto Nacional de Sanidad.
- 1989. MINISTERIO DE SANIDAD Y CONSUMO: Microbiología alimentaria: Detección de bacterias con significado higiçénico-sanitario. Instituto de Salud Carlos III.
- 1992. Intoxicaciones alimentarias de etiología microbiana. R.Eley. Acribia.
- Métodos de análisis microbiológicos de los alimentos. Corrie Allaert, Marta Escolá, Díaz de Santos, 2002
- R.D. 3484/2000. Normas de higiene para elaboración, distribución y comercio de comidas preparadas
- EU DIRECTIVE 2073/2005 of 15 November concerning Microbiological criteria for foodstuffs. The Official Journal of the European Union , 22.12.2005
- 2008 Recopilación de Normas microbiológicas de alimentos. Manuel Moragas (Ayuntamiento de Bilbao) y Mª Begoña de Pablo (Sanidad del Gobierno Vasco).
- ISO 7218. Microbiology of foodstuffs. General rules for microbiological analysis.
- UNE-EN ISO 6888. Microbiology of food and animal feeding stuffs, specifying a horizontal method for the enumeration and detection of coagulase-positive staphylococci (Staphylococcus aureus and other types).
- UNE-EN 12824. Microbiology of food and animal feeding stuffs. Horizontal method for the detection of Salmonella.. (ISO 6579:1993, modified)
- UNE-EN ISO 21567. Microbiology of food and animal feeding stuffs - Horizontal method for the detection of Shigella spp.
- ISO 10272. Microbiology of food and animal feeding stuffs-Horizontal methods for the detection and enumeration of Campylobacter spp.
- UNE-EN ISO 11290. Microbiology of food and animal feeding stuffs-Horizontal methods for the detection and enumeration of *Listeria monocytogenes*.
- UNE-EN 13401.. Microbiology of food and animal feeding stuffs-Horizontal methods for the detection and enumeration of *Clostridium perfringens*. Colony-count technique (UNE-EN ISO 7937 modified)
- UNE-EN-ISO 21871. Microbiology of food and animal feeding stuffs-Horizontal methods for the detection and enumeration of determination of low numbers of presumptive *Bacillus cereus*
- UNE-EN ISO 7932. Microbiology. A general guide for the enumeration of Bacillus cereus. Colony-count technique at 30°C.
- ISO 16649. Routine method for the confirmation of beta-glucuronidase-positive E. coli in food and animal feedingstuffs.
- ISO 16654. Food microbiology. Horizontal methods for the detection of *Escherichia coli* O157.
- ISO 8914. Microbiology of food and animal feeding stuffs. General guide for the detection of *Vibrio parahaemolyticus*.
- UNE-EN ISO 4833. Microbiology of food and animal feeding stuffs-Horizontal methods for the detection of de microorganisms. Colony-count technique at 30 ºC.
- ISO 10273. Microbiology of food and animal feeding stuffs-Horizontal methods for the detection and enumeration of presumptive pathogenic *Yersinia enterolitica*.
- ISO 4832. Microbiology of food and animal feeding stuffs-Horizontal methods for the enumeration of total coliforms. Colony-count technique.
- ISO 21528. Microbiology of food and animal feeding stuffs-Horizontal methods for the detection and enumeration of Enterobacteriaceae
- ISO 7954. Microbiology of food and animal feeding stuffs-Horizontal methods for the enumeration of Yeasts and Moulds
- Sanchis, J. Estudio Intercolaborativo entre los distintos Broths de cultivo generales. XI Congreso Nacional de Microbiología de los Alimentos. Pamplona. 09/1998.
- Sanchis, J. Comparación entre los diversos medios comerciales para aislamiento de hongos (levaduras y mohos): Excelencia del Rosa Bengala Caf.Agar. Técnicas de Laboratorio, Nº 211. 05/1996
- Santos, C.J., Araujo, M., Gómez, M.J., Garrido, M.J.- Evaluación de medios de cultivo para la detección de *Escherichia coli* en aguas. XVII Congreso de la Sociedad Española de Microbiología. Granada. 09/1999
- Validación del medio cromogénico de MICROKIT ® para Salmonella (Chromosalm) mediante un estudio intercolaborativo. Jorge Sanchis Solera, Laboratorios MICROKIT y otros 14 autores. TECNICAS DE LABORATORIO 281, Mayo 2003.
- SEILALIMENTOS reports 1 to 42 (total 1,700 pages), Laboratorios MICROKIT, March-1999 to June-2009
- PRT-SEILA-001: Protocolo GLOBAL **VALIDADO** para la ejecución correcta de análisis de alimentos (e intercomparativos SEILALIMENTOS) (42 páginas)
- PNT-AL-001: Alimentos: Recuento de *Listeria monocytogenes* (17 páginas)
- PNT-AL-002: Alimentos: Investigación de *Listeria monocytogenes* (18 páginas)
- PNT-AL-003: Alimentos: Recuento de microorganismos a 30 ºC (50 páginas)
- PNT-AL-004: Alimentos: Recuento de *E.coli* ß-glucuronidasa positiva (48 páginas)
- PNT-AL-010: Alimentos: Investigación y Recuento de Enterobacterias (60 páginas)
- PNT-AL-016: Alimentos: Investigación y Recuento de *Bacillus cereus* (51 páginas)
- PNT-AL-017: Alimentos: Investigación de *Vibrio parahaemolitycus* (53 páginas)
- PRT-AL/AG-020: Análisis microbiológico del ambiente y operarios en industria agroalimentaria (61 págs)
- PRT-VAL-001 Protocolo para VALIDACIÓN en microbiología (69 páginas)
- PRT-VAL-1+2, Idem, incluido CD con hojas de cálculo en Excel.
- Informes múltiples de validación AOAC, Microval y NordVal sobre las CompactDryPlates® disponibles en Laboratorios Microkit, S.L.