Comparison of NMKL no 136 and ISO 11290 methods		
	NMKL method no 136	ISO 11290
Title	*Listeria monocytogenes*. Detection in foods and feeding stuffs and enumeration in foods	Microbiology of food and animal feeding stuffs-Horizontal method for the detection and enumeration of *Listeria monocytogenes*. **New title proposed** by the ISO/TC 34/SC9 WG Listeria: Microbiology of food and animal feeding stuffs-Horizontal method for the detection and enumeration of *Listeria monocytogenes* **and other *Listeria* spp**.
Amendments		2002: Part1: Detection method Amendment 1: Modification of the isolation media, of the haemolysis test and inclusion of precision data. 2004: Part 2: Enumeration method Amendment 1: Modification of the enumeration medium.
Scope	Detection in foods and feeding stuffs and enumeration in foods Comment: the method has been used and it is applicable also for environmental samples	Detection and enumeration in food and feeding stuffs. (ISO/TC 34/SC9 WG Listeria proposed **to include environmental samples** in the method) and **to include other *Listeria* spp**.
	Qualitative detection	
Principle	Primary enrichment: Half-Fraser broth at 30°C for 24 h. Secondary enrichment: Fraser broth at 37°C for 48 h. The cultures obtained from both the enrichment steps are plated out on a *L. monocytogenes* specific isolation medium, Agar *Listeria* according to Ottaviani and Agosti (ALOA*) or Listeria monocytogenes blood agar medium (LMBA) or Chromogenic Listeria Agar medium basically alike ALOA and on another solid selective isolation medium, which is optional.	Primary enrichment: Half-Fraser broth at 30°C for 24 h. Secondary enrichment: Fraser broth at 35°C or 37°C for 48 h. The cultures obtained from both the enrichment steps are plated out on a *L. monocytogenes* specific isolation medium, Agar *Listeria* according to Ottaviani and Agosti (ALOA) and on another solid selective isolation medium, which is optional.
ALOA enrichment supplement:	2.0 g of L-α-phosphatidyl inositol	2.0 g of L-α-phosphatidyl inositol Proposal by the ISO/TC 34/SC9 WG Listeria: **replacement of phosphatidyl inositol by lecithin**
Pre enrichment	25 g sample (other sample amount than 25 g: dilution rate should be 1:10) add 225 ml Half-Fraser broth Incubate the suspension at 30.0±1.0°C for 24±3 h.	Initial suspension: x g or ml sample to 9x ml or g of the selective primary enrichment (Half-Fraser). Incubate the suspension at 30°C for 24±2 h.
Enrichment	Transfer 0.1 ml of the primary enrichment culture to 10 ml of Fraser broth. Incubate at 37.0±1.0°C for 48±4 h.	Transfer 0.1 ml of the primary enrichment culture to 10 ml of Fraser broth. Incubate at 35°C or 37.0±1.0°C for 48±4 h.
Plating	Both Half- and Fraser are plated out. Streak a loopful (10 µl) of the cultures on the surfaces of **ALOA plates or LMBA plates or Chromogenic Listeria Agar plates** so, that well-isolated colonies are obtained. Proceed in the same way with the optional selective plating medium.	Both the primary and the secondary enrichment cultures are plated out. Streak a loopful (10 µl) of the cultures on the surfaces of **ALOA plates** so, that well-isolated colonies are obtained. Precede in the same way with the optional any other solid selective plating medium.
Incubation	ALOA: 24±3 h at 37.0±1.0°C. If growth is slight or no colonies or no typical colonies re-incubate the plates for further 24±3 h. LMBA: 24±3 h at 37.0±1 and further 24±3 h if growth is slight or no colonies or no typical colonies are observed. Chromogenic Listeria agar plates according to the manufacturer's instructions.	ALOA: 24±3 h at 37.0±1.0°C and if necessary further 24±3 h. Second selective media: appropriate time depending of on the type of media used.
Reading	ALOA After incubation for 24±3 h, and for further 24±3 h, if growth is slight or no colonies or no typical colonies are observed after 24±3 h,	ALOA After incubation for 24±3 h, and for further 24±3 h, if growth is slight or no colonies or no typical colonies are observed after 24±3 h,

	examine the dishes for the presence of colonies presumed to be *L. monocytogenes.* The characteristic colonies of *L. monocytogenes* are green-blue, surrounded by an opaque halo. <u>LMBA</u> After incubation for 24±3 h, examine the dishes for the presence of colonies presumed to be *L. monocytogenes.* After 24 h characteristic colonies are small (about 1 mm in diameter), white-greyish, surrouded by narrow β-haemolysis zone. If growth is slight or no colonies or no typical colonies are observed after 24±3 h, incubate the plates for an additional 24 h±3 h. After 48 h the colonies become larger and the haemolysis broader compared to 24 h incubation. <u>Chromogenic Listeria agar plates</u> Read the Chromogenic Listeria agar plates according to the manufacturer's instructions. Follow the manufacturer's instructions for the reading of the second selective plating medium.	examine the dishes for the presence of colonies presumed to be *L. monocytogenes.* The characteristic colonies of *L. monocytogenes* are green-blue, surrounded by an opaque halo.
nfirmation	Traditional confirmation or validated commercial identification tests can be used. <u>Haemolysis:</u> (calf, bovine, sheep or horse blood) Streak at least **5 colonies** (if available) assumed to be *L. monocytogenes,* **from each selective medium** onto BA. ß-haemolytic colonies from BA presumed to be *L. monocytogenes* are further confirmed. If *L. monocytogenes* is detected after Half-Fraser enrichment, confirmation from Fraser broth could be left out. Confirm at least one pure culture considered being typical or suspecting on BA and four further stepwise until positive *L. monocytogenes* is obtained. <u>Traditional confirmation tests:</u> Confirmation tests are carried out using pure cultures on BA or on TSA. Streak presumptive *L. monocytogenes* colonies onto non-selective agar medium without blood e.g. onto TSA to get pure culture with separate colonies. Incubate the agar at 37.0±1.0°C for 24±3 h NOTE – Catalase test should be carried out from TSA, if the test is performed by dropping the reagent directly on the plate, because in that case media containing blood may cause false positive reactions. <u>Catalase test – suggested to be optional</u> <u>Gram staining</u> can be used, but is not necessary. <u>Carbohydrate metabolism</u> rhamnose and xylose	<u>Haemolysis: DAM.1</u> Can also be carried out using sheep red blood corpuscies. <u>Traditional confirmation tests:</u> Confirmation tests are carried out using pure cultures on BA or on TSA. Streak presumptive *L. monocytogenes* colonies onto non-selective agar medium without blood e.g. onto TSA to get pure culture with separate colonies. Incubate the agar at 37.0±1.0°C for 24±3 h NOTE – Catalase test should be carried out from TSA, if the test is performed by dropping the reagent directly on the plate, because in that case media containing blood may cause false positive reactions. <u>Catalase test</u> <u>Gram staining</u> can be used, but is not necessary. CAMP test <u>Carbohydrate metabolism</u> rhamnose and xylose ISO/TC 34/SC9 WG Listeria proposed: ***L. monocytogenes*: purify 5 colonies from each plate on Blood Agar (haemolysis) and confirm by: Gram stain (optional), Rhamnose and Xylose. Confirm 5 colonies stepwise until a positive result is obtained. *Listeria* spp. ALOA media; purify on Blood agar; confirm: Gram stain (optional), Rhamnose, Xylose and Voges-Proskauer (VP), manitol (*L. grayi*).**
Expression of results	Report the presence/absence of *L. monocytogenes* in 25 g sample (amount of sample analysed). Also specify the method of analysis including the solid selective media used, any deviations from the expected reaction pattern or other data, which may be of relevance for the result and, if needed, sero-, phage- and/or molecular type.	Report the presence/absence of *L. monocytogenes* in test portion, specifying the mass in grams or the volume in millilitres of the sample tested.
Validated	YES. NMKL Report 2006.	Part 1:1996 without the amendment 1 – YES:

		study performed in 1998, EU project SMT4-CT962098, i.e. media tested were Oxford and PALCAM. ALOA and LMBA (original formula) were used by one laboratory (different labs) on voluntary basis.
Performance characteristics of the method	The results of the collaborative study of the detection part are summarised in Table 1 in Annex 1 of the method. Based on the results ALOA or a medium with equal composition, LMBA or Chromogenic Listeria Agar medium basically alike ALOA could be alternatively used as an obligatory isolation medium for the detection of *L. monocytogenes* from foods and from animal feedstuffs.	Precision data by Oxford and PALCAM presented in the amendment 1. Report EU project SMT4-CT962098; EN ISO 11290 Part 1: 1997 (1999). The results by ALOA and LMBA were better compared to the former ones.
Enumeration		
Principle	Initial suspension is incubated for 1 h at 20°C either in buffered peptone water or in the Half-Fraser base to resuscitate stressed mircoorganisms. After the resuscitation period the initial suspension and/or its dilutions are surface plated on a *L. monocytogenes* specific isolation medium, ALOA or LMBA or Chromogenic Listeria Agar medium basically alike ALOA, **in duplicates**. After incubation presumptive *L. monocytogenes* colonies are counted and confirmed using appropriate morphological and biochemical tests. *ALOA is an example of a suitable medium available commercially. The use of other media with the same formulation is allowed.	Preparation of initial suspension (x g or ml) is incubated for 1 h at 20°C either in buffered peptone water or in the Half-Fraser base (9xg or ml) to resuscitate stressed mircoorganisms. After the resuscitation period the initial suspension and/or its dilutions are surface plated on a *L. monocytogenes* specific isolation medium, ALOA **in duplicates**. After incubation presumptive *L. monocytogenes* and *Listeria* spp. colonies are counted and confirmed using appropriate morphological and biochemical tests. ISO/TC 34/SC9 WG Listeria **proposed to remove duplicates.**
Pre-treatment and dilution Resuscitation	Use either BPW or Half-Fraser broth base without addition of the selective agents as diluent for preparing the initial suspension. When Half-Fraser broth base is used, both the detection and the enumeration can be carried out on the same test sample. The selective agents are added to the suspension once the test portion for enumeration has been taken. Take aseptically 10 g sample and transfer to a stomacher bag. Add 90 ml BPW or Half-Fraser broth base. Let this initial suspension stand for 1 h ± 5 min at 20 ±2 °C (if necessary, by using the incubator; in that case the temperature tolerance being ±1°C), in order to resuscitate the stressed microorganisms. If a dilution range is used, prepare it after resuscitation.	Use either BPW or Half-Fraser broth base without addition of the selective agents as diluent for preparing the initial suspension. When Half-Fraser broth base is used, both the detection and the enumeration can be carried out on the same test sample. The selective agents are added to the suspension once the test portion for enumeration has been taken. Take aseptically 10 g sample and transfer to a stomacher bag. Add 90 ml BPW or Half-Fraser broth base. Let this initial suspension stand for 1 h at 20°C, in order to resuscitate the stressed microorganisms. If a dilution range is used, prepare it after resuscitation. ISO/TC 34/SC9 WG Listeria **proposed to remove resuscitation. Preparation of the homogenate with ISO6687.**
Plating	It is necessary to estimate low numbers of *L. monocytogenes* and therefore the limit of enumeration is to be lowered by a factor of 10 by examining 1.0 ml of the resuscitated initial suspension on ALOA or LMBA or Chromogenic Listeria Agar medium. Distribute the 1 ml of inoculum either on the surface of the agar medium in a large Petri dish (140 mm) or over the surface of the agar medium in three small dishes (90 mm). Two small dishes can be used instead of three. In that case agar medium should be dried in the incubator to be able to absorb the inoculum. In all the cases, prepare duplicates. In addition, transfer by means of a sterile pipette 0.1 ml of the resuscitated initial suspension to each of two dishes of ALOA	Distribute the 0.1 ml of initial suspension either on the surface of the agar medium. Dried beforehand if necessary in the incubator. Repeat procedure using further decimal dilutions if necessary. Incubate ALOA plates inverted in an incubator at 35°C or 37.0°C, for 24 h, and for additional 18 h to 24 h.

	or LMBA or Chromogenic Listeria Agar medium, dried beforehand in the incubator, if necessary. Repeat the procedure using further decimal dilutions if necessary. Carefully spread by a sterile spreader the inoculum as quickly as possible over the surface of the agar plate. Leave the plates, lids closed, for about 15 min at ambient temperature for the inoculum to be absorbed into the agar. Incubate plates inverted in an incubator at 37.0±1.0°C, ALOA for the minimum of 36 h, but preferably for 48 ± 4 h and LMBA for 48 h, to get reliable counts. Chromogenic Listeria Agar plates are incubated according to the manufacturer's instructions.	
Counting	ALOA After incubation for 36 h examine the dishes for the presence of colonies presumed to be *L. monocytogenes.* If growth is slight or if no colonies or no typical colonies are present after 36 h of incubation, re-incubate the plates for further 12±2 h. comment: 36 h un-practical, 48 suggested The characteristic colonies of *L. monocytogenes* are green-blue, surrounded by an opaque halo. The incubation period of 48 hours might be needed for the formation of the halo around the colonies of *L. monocytogenes* and for reliable count. Count all the colonies presumed to be *L. monocytogenes* on each dish containing less than 100 characteristic or non-characteristic colonies. LMBA After incubation for 48±4 h examine the dishes for the presence of colonies presumed to be *L. monocytogenes.* After 48 h characteristic colonies are 1-2 mm in diameter, white-greyish, surrounded by narrow β-haemolysis zone. Count all the colonies presumed to be *L. monocytogenes* on each dish containing less than 150 characteristic or non-characteristic colonies. Chromogenic Listeria Agar Count according to the manufacturer's instructions.	After incubation for 24 h and additional 18 h to 24 h count all colonies presumed to be *Listeria* spp. on each dish containing less than 150 characteristic or non-characteristic colonies.
Confirmation	Take the colonies from each countable dish, from each replicate and if possible, from two successive dilutions. Confirm all the typical or suspected pure cultures Perform the same confirmation tests as for detection of *L. monocytogenes* (haemolysis, chatalase, gram staining – optional, carbohydrate metabolism: rhamnose and xylose Comment: Five colonies were confirmed for haemolysis but only one haemolytic colony per sample and per medium were furtherer confirmed in the collaborative study. -> decrease of the number of colonies further confirmed suggested	Select five presumptive ***Listeria* spp** colonies from all dilutions and two successive dilutions; streak on TSYE and confirm by: catalase, gram staining, motility test (if necessary) carbohydrate utilisation, CAMP test. ISO/TC 34/SC9 WG Listeria proposed: ***L. monocytogenes*: purify 5 colonies from each dilution on Blood Agar (haemolysis) and confirm by: Gram stain (optional), Rhamnose and Xylose. Confirm 5 colonies stepwise until a positive result is obtained. *Listeria* spp. ALOA media; purify on Blood agar; confirm: Gram stain (optional), Rhamnose, Xylose and Voges-Proskauer (VP), manitol (*L. grayi*). proposed to purify 5 colonies from each dilution on Blood**

		Agar (haemolysis) and confirm by: Gram stain (optional), Rhamnose and Xylose.
Expression of results	Calculate the concentration (CFU/g or ml) of *L. monocytogenes* from the colony count of confirmed colonies and the degree of dilution. Report the result according to the guidelines described in NMKL Report No. 5. Also specify the method of analysis including the medium used, any deviations from the expected reaction pattern or other data which may be of relevance for the result and, if needed, sero-, phage-and/or molecular type.	See ISO 7218
Validated	YES. NMKL Report 2006.	Part 2:1998 without the amendment 1 – YES; study performed in 1998, EU project SMT4-CT962098, i.e. medium tested was PALCAM, ALOA not included. However, LMBA and Oxford used by one laboratory (different labs) on voluntary basis.
Performance characteristics of the method	The results of the collaborative study of the enumeration part of the method are presented in Tables 1 and 2 in Annex 2. Based on the results ALOA or a medium with equal composition, LMBA or Chromogenic Listeria Agar medium basically alike ALOA could be alternatively used as solid selective plating medium in the enumeration of *L. monocytogenes* from foods. The method cannot be recommended to be used for the enumeration of *L. monocytogenes* from animal feedstuffs.	Precision data not presented in the amendment 1: the results for *L. monocytogenes* were poor. Incorporation of the precision data into the amendment clearly stating that it relates to *Listeria* spp, and revision of the method was recommended to CEN. Report EU project SMT4-CT962098; EN ISO Part 2: 1997 (1999).

Conclusions:

1) NMKL method No. 136 and ISO 11290 with the amendments

The methods can be considered as equal for the detection and enumeration of *L. monocytogenes* from food and feed samples.

2) NMKL method No. 136 and the proposed revision of ISO 11290 (ISO/TC 34/SC9 WG Listeria)

The methods cannot be regarded as equal:

- NMKL-method does not include detection and enumeration other *Listeria* spp. than *L. monocytogenes* that has been proposed by ISO/TC 34/SC9 WG Listeria for revised ISO 11290.
- The WG also proposed replacement of phosphatydil-inositol (PI) by lecithin in revised ISO method. ALOA media suggested in NMKL method contain phosphatydil-inositol (PI). (However, based on the comparisons performed, hardly this causes any differences between the results of ISO and NMKL either in the detection or enumeration of LMO, when the incubation period was prolonged to 48 h in the collaborative study by NMKL.
- NMKL method for the enumeration contains the resuscitation step, which is proposed to be left out from the ISO method.
- The WG proposed removal of duplicate plates from enumeration part in revised ISO 11290.