AL short protocol / Agar

(Agar *Listeria* according to Ottaviani and Agosti)

356-3695 / 356-3965
356-4041 / 356-4042
356-4043 / 355-5200
356-4201

DEFINITION

Selective **chromogenic** medium used for the detection and enumeration of ***Listeria monocytogenes*** and **other species of *Listeria*** in food products for human and animal consumption, and in environmental samples.
This medium may be used in a protocol described in the specifications or in a short protocol validated as an alternative method to the reference methods.

NF VALIDATION by AFNOR CERTIFICATION as per EN ISO 16140 protocol

The AL short protocol method has been certified by NF VALIDATION as an alternative to the reference standard NF EN ISO 11290-1, according to the ISO 16140 protocol, for the **detection of *Listeria monocytogenes* and other species of *Listeria* in all food products for human consumption and in environmental samples.**

BRD 07/16 – 01/09
ALTERNATIVE ANALYTICAL METHODS FOR AGRIBUSINESS
Certified by AFNOR Certification
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The AL short protocol has also been certified by NF VALIDATION as an alternative to the reference standard NF EN ISO 11290-2, according to the ISO 16140 protocol, for the **enumeration of *Listeria monocytogenes* in all food products for human consumption** and in environmental samples.

BRD 07/17 -01/09
ALTERNATIVE ANALYTICAL METHODS FOR AGRIBUSINESS
Certified by AFNOR Certification
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End of NF VALIDATION: please see the certificate BRD 07/16 – 01/09 for the detection protocol and BRD 07/17 -01/09 for the enumeration protocol. These certificates are available from Bio-Rad representative or AFNOR Certification

STANDARDS

- **U.S. Department of Health and Human Services U.S. Food and Drug Administration Center for Food Safety & Applied Nutrition –** Bacteriological, Analytical online Manual, January 2003.

- **NF EN ISO 11290-1/A1 (February 2005):** Food microbiology - Horizontal method for detection and enumeration of *Listeria monocytogenes* - Part 1: Detection method (IC: V08-028-1).
- **NF EN ISO 11290-2/A1 (February 2005):** Food microbiology - Horizontal method for detection and enumeration of *Listeria monocytogenes* - Part 2: Enumeration method (IC: V08-028-2).

PRINCIPLE

The principle of AL medium (Agar Listeria according to Ottaviani and Agosti) is based on the simultaneous detection of 2 enzyme activities: b-glucosidase and phosphatidylinositol-specific phospholipase C (PI-PLC).
β-D-glucosidase activity, common to all *Listeria* genus bacteria is detected using a chromogenic substrate (X-glucoside). Its hydrolysis induces the formation of a blue to blue-green colour in all Listeria colonies.
PI-PLC is an enzyme only detected in pathogenic Listeria species: *L. monocytogenes* and *L. ivanovii*. AL medium contains phosphatidylinositol which, when it breaks down, produces an opaque halo around colonies of bacteria of these 2 species.
This halo generally appears after 24 hours of incubation in *L. monocytogenes* and after only 48 hours of incubation in *L. ivanovii*.
Selectivity of the medium is achieved by the combined action of lithium chloride, antibiotics and the anti-fungal.

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PRESENTATION

Dehydrated

- 500g **code 356-4043**
- Suppl. 1 qsp 500ml (10 vials) **code 356-4041**
- Suppl. 1 qsp 250ml (100 capsules) **code 356-4201**
- Suppl. 2 qsp 500ml (10 x 25ml) **code 356-4042**

Base in bottles (to be supplemented)

- 6 vials of 237.5 ml **code 355-5200**

Pre-poured

- 20 dishes x 90 mm **code 356-3695**
- 120 dishes x 90 mm **code 356-3965**

STORAGE/SHELF-LIFE/BATCH

- Pre-poured and supplements: + 2-8°C protected from light.
- Dehydrated: +15-25°C, in carefully-sealed bottles in a cool, dry place.
- Expiration date and batch number are shown on the package.

THEORETICAL FORMULA

Pre-poured

Meat peptone	18 g
Tryptone	6 g
Yeast extract	10 g
Sodium pyruvate	2 g
Glucose	2 g
Anhydrous magnesium glycerophosphate	1 g
Anhydrous magnesium sulphate	0.5 g
Sodium chloride (NaCl)	5 g
Lithium chloride (LiCl)	10 g
Anhydrous Na_2HPO_4	2.5 g
Chromogenic substrate (5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside)	0.05 g
Nalidixic acid	0.02 g
Ceftazidime	0.02 g
Polymyxin B sulphate	76700 U
Cycloheximide	0.05 g
Phosphatidylinositol	2 g
Agar	12 g

Final $pH_{25°C} = 7.2 \pm 0.2$

PREPARATION FOR :

0 AL SUPPLEMENTS

AL supplement 1 Vial qsp 500 ml (356-4041)

Wearing latex gloves, aseptically reconstitute the contents of the vial with 5 ml of sterile distilled water using a sterile pipette.

AL supplement 1 capsule qsp 250 ml (356-4201)

Ready to use. Wearing latex gloves aseptically open the capsule and add its content in one vial of AL base (code 355-5200) or to 237.5 ml of AL medium reconstituted from the dehydrated base (code 356-4043).

AL supplement 2, 25ml, 10 vials qsp 500ml (356-4042)

Pre-heat the supplement to 44-47°C for at least 5 minutes in a water bath.

0 DEHYDRATED MEDIUM

Always shake before use

Dehydrated base

Dissolve **34.55 g** of powder in **470 ml** of distilled water. To ensure the powder is dispersed, stir for 10 minutes. Sterilise by autoclaving for 15 minutes at 121°C.

500 g of powder makes 7.2 liters of medium

Complete medium

After autoclaving, cool the base to 44-47°C and add the **AL supplement 1 qsp 500 ml** (356-4041) in **470 ml of base**, swirling to mix. Then add the vial of **AL supplement 2 pre-heated** to 44-47°C and disperse with gentle end over end mixing before pouring into Petri dishes**.**

Warning : the temperature of the base should not exceed 50°C when adding the supplement 2. There is a risk of precipitation in the medium. Stack the dishes (not more than 5) to allow slow cooling and then dry the agar surface well.

0 READY TO USE BASE

Complete medium

Smelt the AL base medium (code 355-5200) and cool to 44-47°C. Add **AL supplement 1**:

2.5 ml of AL supplement 1 qsp 500 ml (356-4041) reconstituted, **or 1** AL supplement capsule qsp 250 ml (356-4201). Swirling to mix. Add **12.5 ml** (half a vial) of AL supplement 2 (356-4042) **pre-heated** to 44-47°C and disperse with gentle before pouring into Petri dishes.

Warning: the temperature of the base should not exceed 50°c when adding the supplement 2. Stack Petri dishes together (e.g. 5) to allow slow cooling and then dry the agar surface well.

OTHER PRODUCTS REQUIRED (NOT SUPPLIED)

- Tryptone Salt diluent:

9 ml x 25 tubes	(e.g.* **code 355-5754)**
90 ml x 6 bottles	(e.g.* **code 355-5756)**
500 g	(e.g.* **code 356-4544)**
5 x 2.3 l bags	(e.g.* **code 355-5791)**

Detection method:

- FRASER 1/2 broth:

Ready-to-use (complete):

6 x 225 ml bottles	(e.g.* **code 355-5797)**
4 x 3 l bags	(e.g.* **code 355-5794)**
2 x 5 l bags	(e.g.* **code 355-5792)**
Dehydrated Base 500 g	(e.g.* **code 356-4604)**

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Freeze-dried selective supplement:
Pack of 10 bottles **(e.g.* code 356-4616)**

Enumeration method:
• FRASER 1/2 broth without selective agents with ferric (III) ammonium citrate
• FRASER 1/2 broth:
Ready-to-use (complete):
6 x 225 ml bottles **(e.g.* code 355-5797)**
4 x 3 l bags **(e.g.* code 355-5794)**
2 x 5 l bags **(e.g.* code 355-5792)**
Dehydrated
Base 500 g **(e.g.* code 356-4604)**
Freeze-dried selective supplement: Pack of 10 bottles **(e.g.* code 356-4616)**

• Buffered peptone water:
6 x 225 ml bottles **(e.g.* code 355-4179)**
500 g **(e.g.* code 356-4684)**
4 x 3 l bags **(e.g.* code 355-5795)**
2 x 5 l bags **(e.g.* code 355-5790)**

Confirmation
• RAPID'*L.mono* agar
20 dishes x 90 mm (**code 356-3694**)
120 dishes x 90 mm (**code 356-3964**)
Ready-to-use: 1 pack (**code 355-5294**)

Dehydrated + supplements
500g (**code 356-4293**)
Supplement 1 (10 flasks, to make 500 ml of basic agar) (**code 356-4294**)
Supplement 2 (10 flasks, to make 500 ml of basic agar) (**code 356-4746**)

• PALCAM
Pre-poured
20 dishes x 90 mm (e.g.* **code 356-3674**)

Dehydrated + supplement
500g (e.g.* **code 356-4754**)
Supplement (e.g.* **code 356-4752**)

See corresponding Technical Sheet(s)

MATERIALS REQUIRED NOT PROVIDED (non-exhaustive)

- Scales
- Sterile weighing bags
- Mill
- Stirrer-homogeniser
- Sterile Petri dishes (Ø = 90 or 140 mm)
- Sterile pipettes (0.1 ml; 1 ml…..)
- Sterile spreaders
- Sterile Pasteur pipettes
- Water-bath precise to ± 1 °C
- Thermostatically-controlled incubator or incubation room precise to ± 1 °C
- All usual laboratory equipment

*e.g. = for example

ALTERNATIVE PROTOCOLS

• *Listeria monocytogenes* and other *Listeria* detection in samples as per short protocol:

• Preparation of sample/ Enrichment

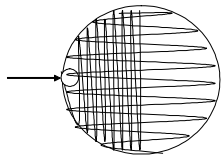
- Dilute n g or n ml of sample in 9 x n ml of Fraser ½ broth.
- Incubate at 30°C (± 1°C) for 24 h (± 2 h).

NB: After incubation, Fraser ½ selective enrichment broth may be stored in a refrigerator (2-8°C) for 72h, before the inoculation of the AL agar.

Note: In the context of NF VALIDATION mark, no samples of over 25 g were tested.

• Isolation and Incubation

- Pipette 0,1mL from the inoculated half Fraser broth and lay it as a drop onto the surface of an AL agar plate close to the side of the plate. Streak this inoculum with a loop on half of the plate, than streak on the other half of the plate coming back onto the first half as described in the scheme bellow:



- Incubate the upturned dishes at 37°C (± 1°C) for 24 h (±2 h)

• Reading

- Take a reading of the *Listeria monocytogenes* after 24 hours (± 2 h) of incubation.

Note 1: It is possible to take a reading of the *Listeria monocytogenes* after 48 hours of AL agar incubation.

Note 2: After incubation, the AL agar dishes can be stored in a refrigerator (2-8°C) for 72 hours, before reading and confirmation.

Note 3: In case of a positive result with the AL detection validated method, it is not necessary to make a confirmation if the sample has been already confirmed as positive for *Listeria monocytogenes* in the enumeration method.

• Enumeration of *Listeria monocytogenes* as per short protocol

• Preparation of sample/Revivification

- Inoculate the sample in Fraser 1/2 complete broth or buffered peptone water (1/10

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dilution).
- Incubate at 20 °C (± 2 °C) for 1 h (± 5 min)
-> stock solution

NB: the revivification step in Buffered peptone water is optional

• Spreading and Incubation
Using stock solution:
- Spread 0.1 ml on 1 dish of AL agar on the surface or 1ml in inclusion in one dish of AL agar (in-depth inoculation)
- Incubate the upturned dishes at 37°C (± 1°C) for 48± 3 hours.

Note: If for some products it is necessary to estimate small numbers, spread 1 ml of stock solution over 3 dishes (~ 0.33 ml/dish) of AL agar or 1ml in inclusion in one dish of AL agar (in-depth inoculation)

• Reading
- A first reading at 24h allows a more rapid detection of heavily contaminated samples. However the final result count is reached after 48 h+/- 3h.

NB 1: After incubation, the AL agar dishes can be stored in a refrigerator (2-8°C) for 72 hours, before reading and confirmation.

NB 2: In case of a positive result with the AL enumeration validated method, it is not necessary to make a confirmation if the sample has been already confirmed as positive for *Listeria monocytogenes* in the detection method.

NB 3: Confirm less than five colonies involves a risk of making an overestimation because of the presence of typical colonies that would not be *Listeria monocytogenes.*

• Expression of results/Calculations
Refer to standard ISO 11290 part 1 and 2, and NF ISO 7218 for the calculation method.

• Confirmation of characteristic colonies
***Listeria monocytogenes* :**
Blue / blue-green colonies with halo

In the context of NF VALIDATION Mark, only one colony confirmation is required, and must be confirmed in one of the following way:

- Using the **conventional tests** described in the standardised ISO reference methods (with purification step)

- Using **nucleic probes** as described in ISO 7218 standard, using isolated colonies (with or without purification step), for example: **iQ-Check™** *Listeria monocytogenes* PCR method (code **357-8124)**.

- A colony isolated on AL agar may be confirmed by means of **spot sub-culture** on RAPID*'L. mono* agar.
Note: Up to 12 colonies can be confirmed on one dish of RAPID*'L. mono* agar.

- Using any **NF VALIDATION certified method** based on a **different principle** from that of AL agar. The validated second method protocol should be respected in its entirety, i.e. all stages preceding the intermediary stage from which confirmation is sought must be common to the two methods.

***Listeria* spp. :**
Blue / blue-green colonies with or without halo

In the context of NF VALIDATION Mark, one colony confirmation is required, and must be confirmed in one of the following way:

- Using the **conventional tests** described in the standardised ISO reference methods (with purification step)

- Using **nucleic probes** as described in ISO 7218 standard, using isolated colonies (with or without purification step), for example: **iQ-Check™** *Listeria* spp. PCR method (code **357-8113)**.

- A colony isolated on AL agar may be confirmed by **streak sub-culture** on **PALCAM**.
Note: Up to 6 colonies can be confirmed on one dish of PALCAM.

- Using any **NF VALIDATION certified method** based on a **different principle** from that of AL agar. The validated second method protocol should be respected in its entirety, i.e. all stages preceding the intermediary stage from which confirmation is sought must be common to the two methods.

In the event of discrepant results (positive with AL method, negative with confirmation method), the laboratory should take sufficient measures to ensure the validity of its findings.

• Confirmation of *Listeria monocytogenes* using AL medium in the context of

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RAPID'*L.mono* **method certified NF validation (BRD No. 07/04 - 09/98.)**

The method of detection of *Listeria monocytogenes* with RAPID'*L.mono* medium permits confirmation of positive results using AL medium.

- **Isolation and incubation**
- Carry out spot sub-culture of an isolated colony from RAPID'*L.mono* medium directly on AL agar (It is possible to confirm up to 12 colonies on one dish of AL agar)
- Incubate the upturned dishes at 37°C (± 1°C) for 24 hours (± 2 h)

- **Reading**
- Take a reading after 24 hours (± 2 h) of incubation.

STANDARD PROTOCOLS

• Detection of Listeria monocytogenes as per ISO 11290-1/A1 standard:

- **Preparation of sample/ Primary selective enrichment**
- Inoculate η g or η ml of sample on 9 x η ml of Fraser 1/2 broth.
- Incubate at 30°C (± 1°C) for 24 hours (± 2 h).

- **Secondary selective enrichment**
- Inoculate 0.1 ml of Fraser 1/2 broth at the end of incubation on 10 ml of Fraser 1
- Incubate at 37°C (± 1°C) for 48 hours (± 2 h).

- **Isolation and Incubation**
- Using a sterile inoculating loop, take up enrichment broth at the end of incubation (Fraser1/2 or Fraser 1* broth) and isolate on the surface of AL agar.
- Incubate the upturned dishes at 37°C (± 1°C) for 24 h (± 3 h) and, if necessary, for a further 24 h (± 3 h).

* It is not necessary to perform isolation in Fraser 1 broth if characteristic *L. monocytogenes* colonies are already detected with Fraser 1/2 broth.

- **Reading**
- Take a reading after 24 hours and if necessary after 48 hours of incubation.

• Enumeration of Listeria monocytogenes as per ISO 11290-2/A1 standard:

- **Preparation of sample/Revivification**
- Inoculate η g or η ml of sample on 9 x η ml of buffered peptone water or Fraser broth _ without selective agents with ferric (III) ammonium citrate.
- Incubate at 20°C (± 2°C) for 1 h (± 5 min)

-> stock solution

- **Spreading and Incubation**

Using stock solution:
- Spread 0.1 ml on 1 dish of Agar *Listeria* according to Ottaviani and Agosti (AL)
- If necessary, carry out a 1/10 (or more) dilution in Tryptone Salt diluent and spread 0.1 ml of each dilution over 1 dish of AL agar.
- Incubate the upturned dishes at 37°C (± 1°C) for 24 h (± 3 h) and 48 h (± 3 h) .

If, for some products, it is necessary to estimate small numbers, spread 1 ml of stock solution over 3 dishes (~ 0.33 ml/dish) of AL agar.

- **Reading**
- Take a reading after 24 hours and 48 hours of incubation.

Note: Count only dishes containing a maximum of 150 characteristic colonies and a maximum of 300 colonies in total.

• Confirmation of characteristic colonies

For confirmation and identification of suspected *L. monocytogenes* colonies, select 5 characteristic colonies, i.e. blue/blue-green with halo (if there are fewer, retain them all) and perform identification tests according to Standard 11290-1 and 2 summarised in Tables I and II.

Table I:
Test of adherence to *Listeria* genus.

	GRAM	CATALASE	MOBILITY
Listeria **GENUS**	+	+	+

Table II:
Identification of *Listeria monocytogenes*

	1	2	3	4	5	6	7
L.monocytogenes	+	-	+	-	+	-	-

Legend:
1 : HAEMOLYSIS
2 : C.A.M.P. TEST *R. equi*
3 : C.A.M.P. TEST *S. aureus*
4 : D-XYLOSE
5 : L-RHAMNOSE
6 : MANNITOL
7 : NITRATE REDUCTION
+ : over 90 % positive reaction
- : no reaction

- **Expression of results/Calculations**
- Refer to Standards ISO 11290-1 and 2, ISO 7218 and XP V08-102

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PRECAUTIONS

- Standard precautions concerning the handling of potentially-contaminated products in a microbiology laboratory should be observed.
- For heavily-loaded dishes with intensely opaque agar, reading can be facilitated by comparing opacity of the agar with a non inoculated AL dish.
- Other Gram positive β-D-glucosidase positive bacteria exist without halos (e.g. *Enterococcus* spp.) and with halos (e.g. *Bacillus* spp.)
- Beware of slow *L. monocytogenes* β-D-glucosidase-positive strains.
- Before using the AL agar dishes, leave them to dry, according to Standard ISO 7218, at 25°-50°C until the drops on the surface of the medium have disappeared. Prolonged drying can alter the medium's performance, and should be avoided.
- Comply with Good Laboratory Practice(EN ISO 7218)..

PERFORMANCES / QUALITY CONTROL OF THE TEST

The growth performances of the media are verified with the following strains:

STRAINS	Appearance of colonies after 24-48 hours of incubation at 37 °C	
	GROWTH	COLOUR
Listeria innocua WDCM00017	+	Blue Halo (-)
Listeria. monocytogenes 1/2a WDCM00020	+	Blue Halo (+)
Listeria monocytogenes 4b WDCM00021	+	Blue Halo (+)
Escherichia coli WDCM00009	Inhibition	NA
Enterococcus faecalis WDCM00013	Inhibition	NA

NA = Not applicable

QUALITY CONTROL OF MANUFACTURER

Every product manufactured and marketed by Bio-Rad is subject to a quality-assurance procedure at all stages, from reception of raw materials through to marketing of the finished products.

Each batch of finished product undergoes quality control and is marketed only if it satisfies the acceptability criteria.

Documentation relative to the production and quality control of each batch is kept on file.

KEY WORDS

Agar *Listeria* according to Ottaviani and Agosti (AL) / *Listeria monocytogenes* / Detection / Enumeration / Food products / Fraser / Glucosidase / Phospholipase / Chromogenic / Medium.

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