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Authors: Régine Talon (INRA), Sabine Leroy (INRA), Nicolas Ayessou (UCAD), Victor Anihouvi (UAC), Générose Vieira Dalodé (UAC), Victor Jeannoda (UT), Sameh Awad (FAAU), Wisdom Amoa (FRI), Manuela Pintado (ESB), Didier Montet (CIRAD), Gérard Loiseau (CIRAD), Edna Arcuri (CIRAD)

**This document has been send to :**

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**AFTER (G.A n°245025) – Deliverables 1.2.3.4, 1.2.3.5, 1.2.3.6  
SOPs for microbiological methods for Group 1, 2 and 3**

Methodology for the development of SOPs for this deliverable

This deliverable combined 3 deliverables: D1.2.3.4, D.1.2.3.5 and D1.2.3.6. It consists of several SOPs related to the microbiological methods. We decided to present only one deliverable for all group of products because most of the SOPs are common for each group. So in this deliverable you will have all the microbiological methods for group 1, 2 and 3.

The aim of this deliverable is to standardize the methodology for microbiological assessments of the ten food products studied.

For the microbiological assessments of the samples, the microorganisms to study have been listed based on the EU regulation (Règlement CE N° 2073/2005 concernant les critères microbiologiques applicables aux denrées alimentaires).

First the preparation of the samples: initial suspensions and decimal suspensions will be carried out according ISO 6887-1:1999. The preparation will consider each category of products:

- Meat and meat products : ISO 6887-2 : 2004
- Fish and fish products : ISO 6887-3 : 2004
- Milk and milk products : ISO 6887-5 : 2010
- Others : ISO 6887-4 : 2004

The procedure for enumeration and the following microorganisms listed in the table below will be studied in the different products as described in the 10 SOPs based on ISO standards.

These ISO standards have been bought for the different partners by the coordinator.

<b>Microorganisms</b>	<b>Group 1 Cereals Akpan, Gowé, Kenke, Kishk</b>	<b>Group 2 Fish/Meat Lanhouin, Kong Kitoza</b>	<b>Group 3 Plant extract Baobab, Hibiscus, Jaabi</b>	<b>SOP</b>
Samples preparation	+	+	+	Micro-00, ISO 6887-1:1999
Enumeration of microorganisms	+	+	+	Micro-01, ISO 4833
<i>Enterobacteriaceae</i>	+	+	+	Micro-02, ISO 21528-2
<i>Escherichia coli</i>	+	+	+	Micro-03, ISO 16649-2
<i>Bacillus cereus</i>	+	+	+	Micro-04, ISO 7932
<i>Staphylococcus aureus</i> and CPS	+	+	+	Micro-05, ISO 6888-1
<i>Listeria monocytogenes</i>	+	+	-	Micro-06, ISO 11290-1/A1:2004
<i>Salmonella</i>	+	+	-	Micro-07, ISO 6579:2002
<i>Clostridium perfringens</i>	+	+	+	Micro-08, ISO 7937
Yeasts and moulds	+	+	+	Micro-09, ISO 7954
Coagulase Negative Staphylococci (CNS)	-	+	-	Micro-05, ISO 6888-1
Lactic acid bacteria (LAB)	+	+	+	Micro-10, M-METH-MO-13

## List of SOPs for the microbiological methods

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**A. Procedure for preparation of test samples, initial suspension and decimal dilutions for microbiological examination**

(Group 1, 2 and 3)

<b>Procedure for preparation of test samples, initial suspension and decimal dilutions for microbiological examination</b>		
SOP Number: <b>Micro-00</b>		
Date of creation: 03/05/2011	Date of revision: 09/26/2011	
Written by : Marina Rivollier & Edna Froeder Arcuri		
For information on this SOP please contact : Marina Rivollier & Edna Froeder Arcuri		
<b>This document has been approved by :</b>		
Partner	Name of the person who approved	Date:09/26/2011
CIRAD	Didier Montet	
UAC	Victor Anihouvi	
UCAD	Nicolas Ayessou	
FAAU	Sameh Awad	
ESB	Manuella Pintado	
FRI	Wisdom Amoa	

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## 1 Scope and application

This procedure can be applied to Group 1: Cereal based products (Akpan, Kenkey, Gowé, Kishk Sa'eedi) and Group 2: Meat and Fish products (Kizota, Lanhouin, Kong), Group 3: Plant based extract (adansonia digitata, hibiscus sabdariffa, ziziphus mauritana).

## 2 References

NF EN ISO 6887-1: 1999

Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination.

Part 1: General rules for the preparation of the initial suspension and decimal dilutions.

NF EN ISO 6887-2: 2004

Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination.

Part 2: Specific rules for the preparation of meat and meat products.

NF EN ISO 6887-3: 2004

Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination.

Part 3: Specific rules for the preparation of fish and fishery products.

NF EN ISO 6887-4: 2004

Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination.

Part 4: Specific rules for the preparation of products other than milk and milk products,

NF EN ISO 6887-5: 2010

Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination.

Part 5: specific rules for the preparation of milk and milk

*NOTE: All the procedures are described in the standards mentioned above. It's important to mention that for each category of product (i.e. meat and meat products), the **preparation of test samples (Parts 2 to 5)** is explained and detailed taking into account the product specificities (frozen, dry, heterogeneous, acidic, with high fat content...). Thus, it's recommended to refer directly to the appropriated standard.*

*Herein, we only focus the on "**initial suspension and decimal dilutions**" part (Part 1) and more particularly (1) on the item 4 Principle and (2) described some important points at the item 8 Critical points or note of the procedure.*

### 3 Definitions

### 4 Principle

### 5 Diluents

### 6 Apparatus

### 7 Procedure

### 8 Critical points or Note on the procedure

Refer to the item:

#### 7 Procedure

##### - SAMPLE HOMOGENATE or INITIAL DILUTION :

- Each sample should be mixed to ensure homogeneity before aseptically withdrawing a mass of  $m$  g or a volume  $V$  ml (at least 10 g or 10 ml must be taken for the first dilution, except if specified).
- Add exactly nine times volume of diluents at ambient temperature to the weight or volume of sample to give a  $10^{-1}$  suspension (1:9 sample-to-diluent ratio).
- Using a stomacher, homogenize the suspension 2 minutes. Samples which may puncture the stomacher bag must be placed inside a further two or three stomacher bags to prevent leakage.

##### - DECIMAL DILUTION:

- Use diluent solutions at ambient temperature to all dilutions.
- To prepare decimal dilutions transfer 1.0 ml of the  $10^{-1}$  homogenate to 9.0 ml of diluents. Mix carefully using a vortex mixer for 5 -10 s. This constitutes the  $10^{-2}$  dilution. Using a fresh steril pipette/pipette tip for each dilution, repeat this procedure to obtain further decimal dilutions.
- The time lapse between preparation of the homogenate/dilutions and inoculation of the counting media should not exceed 45 minutes. If the ambient temperature of the laboratory is very high it is better to reduce this time.

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SOPs for microbiological methods for Group 1, 2 and 3**

## **9 Test report**

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification for the sample.

## **10 Revision record**

<b>Date</b>	<b>Responsible person</b>	<b>Description of change</b>
09/26/11	Meeting group	



**B. Procedure for enumeration of microorganisms – Colony-count at 30 °C**  
( Group 1, 2 and 3)

<b>Procedure for enumeration of microorganisms – Colony-count at 30 °C</b>		
SOP Number: <b>Micro-01</b>		
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Written by : Edna Froeder Arcuri  For information on this SOP please contact : Edna Froeder Arcuri		
<b>This document has been approved by :</b>		
Partner	Name of the person who approved	Date
CIRAD	Didier Montet	09/26/2011
UAC	Victor Anihouvi	
UCAD	Nicolas Ayessou	
FAAU	Sameh Awad	
ESB	Manuella Pintado	
FRI	Wisdom Amoa	

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- 11 Revision record

## 1 Scope and application

This procedure can be applied to Group 1: Cereal based products (Akpan, Kenkey, Gowé, Kishk Sa'eedi) and Group 2: Meat and Fish products (Kizota, Lanhouin, Kong), Group 3: Plant based extract (adansonia digitata, hibiscus sabdariffa, ziziphus mauritana).

## 2 References

International Standard – NF EN ISO 4833, May 2003  
Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of microorganisms – Colony-count technique at 30 °C.

NOTE: All the procedure is described in the NF EN ISO 4833. We only focus on the item 4 Principle and described some important points at the item 9 Critical points or note of the procedure.

## 3 Definitions

## 4 Principle

Two Petri dishes are inoculated with a specified amount of a liquid sample or primary dilution if it is not a liquid product.

Others Petri dishes are inoculated, at the same conditions, with decimal dilutions obtained from a liquid sample or primary dilution.

The medium is added and mixed (pour plate method).

The plates are incubated under aerobic conditions at 30 °C for 72 h.

The colonies are counted and the number of viable micro-organisms per milliliter or gram of sample is calculated.

## 5 Reagents

## 6 Apparatus

## 7 Procedure

## 8 Expression of results

Method of calculation and formulae

Calculation

Formulae

Repeatability

## 9 Critical points or Note on the procedure

Refer to the items:

### 7 Procedure

- **SAMPLE PREPARATION AND DILUTION :**
- To prepare the decimal dilutions use peptone saline solution or buffered peptone water.
- **COLONY COUTING:**
  - o After the incubation period, select plates containing 15 to 300 colonies. Spreading colonies are considered as single colonies. If less than one quarter of the plate is overgrown by spreading colonies, count the colonies on the unaffected part of the petri plate and calculate the corresponding number of colonies for the entire plate. If more than one quarter of the plate is overgrown by spreading colonies, discard the plate.

### 8 Result expression:

- o Record the count as two significant numbers and expressed as a power of 10. When the third number is less than five, do not change the preceding number; when the third number is five or more, increase the preceding number by one unit.
  - Example: 38500 is expressed as  $3,9 \times 10^4$ .

## 10 Test report

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification of the sample.

## 11 Revision record

Date	Responsible person	Description of change
09/26/11	Meeting group	

**C. Procedure for Enterobacteriaceae detection**  
(Group 1, 2 and 3)

<b>Procedure for Enterobacteriaceae detection</b>		
SOP Number: <b>Micro-02</b>		
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Written by : Edna Froeder Arcuri		
For information on this SOP please contact : Edna Froeder Arcuri		
<b>This document has been approved by :</b>		
Partner	Name of the person who approved	Date
		09/26/11
CIRAD	Didier Montet	
UAC	Victor Anihouvi	
UCAD	Nicolas Ayessou	
FAAU	Sameh Awad	
ESB	Manuella Pintado	
FRI	Wisdom Amoa	

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- 10 Test report**
- 11 Revision record**

## 1 Scope and application

This procedure can be applied to Group 1: Cereal based products (Akpan, Kenkey, Gowé, Kishk Sa'eedi) and Group 2: Meat and Fish products (Kizota, Lanhouin, Kong), Group 3: Plant based extract (adansonia digitata, hibiscus sabdariffa, ziziphus mauritana).

## 2 References

NF ISO 21528-2

Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of Enterobacteriaceae – Part 2: Colony-count method.

NOTE: All the procedure is described in the NF ISO 21528-2. We only focus on the item 4 Principle and described some important points at the item 9 Critical points or note of the procedure.

## 3 Definitions

## 4 Principle

### 4.1 Preparation of the primary suspension and decimal dilutions

Preparation of the primary suspension and decimal dilutions of the food sample.

### 4.2 Isolation

*Two Petri dishes are inoculated with a specified amount of a liquid sample or primary dilution if it is not a liquid product.*

Others Petri dishes are inoculated, at the same conditions, with decimal dilutions obtained from a liquid sample or primary dilution.

Violet Red Bile Agar is added (pour plate method) and after solidification, it is added a second layer of the same medium.

The plates are incubated at  $37\text{ °C} \pm 1\text{ °C}$  for  $24\text{ h} \pm 2\text{ h}$ .

### 4.3 Confirmation

Presumptive Enterobacteriaceae colonies are streaked on a medium not selective and confirmed by glucose fermentation and oxidase test.

### 4.4 Calculation

From the number of characteristic colonies confirmed per plate, it is calculated the number of Enterobacteriaceae per gram or ml.

## 5 Reagents

## 6 Apparatus

## 7 Procedure

## 8 Expression of results

### 8.1 Method of calculation and formulae

#### 8.1.1 Calculation

#### 8.1.2 Formulae

### 8.2 Repeatability

## 9 Critical points or Note on the procedure

Refer to the item:

### 7 Procedure

#### - SAMPLE PREPARATION AND DILUTION :

- To prepare sample dilutions use peptone saline solution (PSD) or buffered peptone water.

#### - COUTING AND SELECTING COLONIES FOR CONFIRMATION:

- On Violet Red Bile Agar the colonies are pink to purple-red (surrounded or not by a halo of precipitated bile acids).
- Note: some Enterobacteriaceae may cause discoloration of their colonies or of the medium. Consequently, if no characteristic colony is present, to choose five non characteristic colonies for confirmation.

#### - CONFIRMATION:

- Utilize only pure cultures for confirmation.
- The colonies that ferment glucose and are oxydase positive are considered *Enterobacteriaceae*.



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SOPs for microbiological methods for Group 1, 2 and 3**

## **10 Test report**

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification of the sample.

## **11 Revision record**

<b>Date</b>	<b>Responsible person</b>	<b>Description of change</b>
09/26/11	Meeting group	

## D. Procedure for *Escherichia coli* $\beta$ -glucuronidase positive detection

( Group 1, 2 and 3)

<b>Procedure for <i>Escherichia coli</i> <math>\beta</math>-glucuronidase positive detection</b>		
SOP Number: <b>Micro-03</b>		
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Written by : Edna Froeder Arcuri		
For information on this SOP please contact : Edna Froeder Arcuri		
<b>This document has been approved by :</b>		
Partner	Name of the person who approved	Date
		09/26/11
CIRAD	Didier Montet	
UAC	Victor Anihouvi	
UCAD	Nicolas Ayessou	
FAAU	Sameh Awad	
ESB	Manuella Pintado	
FRI	Wisdom Amoa	

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## 1 Scope and application

This procedure can be applied to Group 1: Cereal based products (Akpan, Kenkey, Gowé, Kishk Sa'eedi) and Group 2: Meat and Fish products (Kizota, Lanhouin, Kong), Group 3: Plant based extract (adansonia digitata, hibiscus sabdariffa, ziziphus mauritana).

## 2 References

NF ISO 16649-2

Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of  $\beta$ -glucuronidase-positive *Escherichia coli*. Part 2: Colony-count technique at 44 °C using 5-bromo-4-chloro-3-indolyl  $\beta$ -D-Glucuronate.

NOTE: All the procedure is described in the NF ISO 16649-2. We only focus on the item 4 Principle and described some important point at the item 9 Critical points or note of the procedure.

## 3 Definitions

## 4 Principle

- *Inoculation, in duplicate Petri dishes, tryptone-bile-glucuronide (TBX) agar with a specified amount of a liquid sample or primary dilution if it is not a liquid product.*
- At the same conditions, inoculation of other decimal dilutions obtained from a liquid sample or primary dilution.
- Incubation of plates at 44 °C  $\pm$  1 h for 18 to 48 h.
- Calculation of the UFC of *Escherichia coli*  $\beta$ -glucuronidase-positive per gram or millilitre of sample, from the number characteristic colonies.

## 5 Reagents

## 6 Apparatus

## 7 Procedure

## 8 Expression of results

### 8.1 Method of calculation and formulae

#### 8.1.1 Calculation

#### 8.1.2 Formulae

### 8.2 Repeatability

## 9 Critical points or Note on the procedure

Refer to the items:

### 7 Procedure

#### INCUBATION

- If the presence of stressed microorganisms is suspected, to incubate initially during 4 h at a temperature of 37 °C, then during 18 h to 24 h at 44 °C. The temperature of incubation should not exceed 45 °C.

## 10 Test report

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification of the sample.

## 11 Revision record

Date	Responsible person	Description of change
09/26/11	Meeting group	

**E. Procedure for *B. cereus* detection**  
(Group 1, 2 and 3)

<b>Procedure for <i>B. cereus</i> detection</b>		
SOP Number: <b>Micro-04</b>		
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Written by : Edna Froeder Arcuri		
For information on this SOP please contact : Edna Froeder Arcuri		
<b>This document has been approved by :</b>		
Partner	Name of the person who approved	Date
		09/26/11
CIRAD	Didier Montet	
UAC	Victor Anihouvi	
UCAD	Nicolas Ayessou	
FAAU	Sameh Awad	
ESB	Manuella Pintado	
FRI	Wisdom Amoa	

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    - 8.1.1 Calculation
    - 8.1.2 Formulae
  - 8.2 Repeatability
- 9 Critical points or Note on the procedure**
- 10 Test report**
- 11 Revision record**

## 1 Scope and application

This procedure can be applied to Cereal based fermented foods (Akpan, Kenky, Gowé, Kishk Sa'eedi), fermented salted fish and meat (Lanhouin, kizota, kong), and to plant based extract (Adansonia digitata, hibiscus sabdariffa, ziziphus mauritana).

## 2 References

NF EN ISO 7932, July 2005

Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of presumptive *Bacillus cereus* – Colony-count at 30 °C.

NOTE: All the procedure is described in the NF EN ISO 7932. We only focus on the item 4 Principle and described some important points at the item 9 Critical points or note of the procedure.

## 3 Definitions

## 4 Principle

- *Two Petri dishes containing the selective agar are inoculated with a specified amount of a liquid sample or primary dilution if it is not a liquid product.*
- *At the same conditions, inoculate the other decimal dilutions obtained from a liquid sample or primary dilution.*
- *The plates are incubated in aerobic conditions at 30 °C for 18 to 48 h.*
- *The number of B. cereus per milliliter or gram of sample are calculated after counting the number of typical colonies from selected plate (s) giving significant result, and confirmed by the specific tests.*



## 5 Reagents

## 6 Apparatus

## 7 Procedure

## 8 Expression of results

### 8.1 Method of calculation and formulae

#### 8.1.1 Calculation

#### 8.1.2 Formulae

### 8.2 Repeatability

## 9 Critical points or Note on the procedure

Refer to the item:

### 7 Procedure

#### - SAMPLE PREPARATION AND DILUTION:

To prepare the decimal dilutions use peptone saline solution or buffered peptone water.

#### - COLONY COUNTING:

- After the incubation period, select plates contain less than 150 colonies, and if possible, plates from two consecutive dilutions for counting. Presumptive colonies of *B. cereus* are large and pink (do not ferment mannitol, see Note 1) and usually surrounded by precipitate zone, which indicates that lecithinase is produced (see Note 2).

Note 1: If the plates have many micro-organisms that produce acid from mannitol, the pink color characteristic of the colonies of *B. cereus* may be faint or disappear completely.

Note 2: Some strains of *B. cereus* produce few or any lecithinase. Therefore, their colonies will not be surrounded by precipitate zone. These colonies must be submitted to the confirmation tests.

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SOPs for microbiological methods for Group 1, 2 and 3**

## **10 Test report**

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification of the sample.

## **11 Revision record**

<b>Date</b>	<b>Responsible person</b>	<b>Description of change</b>
09/22/2010	Meeting group	

**F. Procedure for coagulase-positive staphylococci detection**  
(Group 1, 2 and 3)

<b>Procedure for coagulase-positive staphylococci detection</b>		
SOP Number: <b>Micro-05</b>		
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Written by : Edna Froeder Arcuri		
For information on this SOP please contact : Edna Froeder Arcuri		
<b>This document has been approved by :</b>		
Partner	Name of the person who approved	Date
		09/26/11
CIRAD	Didier Montet	
UAC	Victor Anihouvi	
UCAD	Nicolas Ayessou	
FAAU	Sameh Awad	
ESB	Manuella Pintado	
FRI	Wisdom Amoa	

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## 1 Scope and application

This procedure can be applied to Group 1: Cereal based products (Akpan, Kenkey, Gowé, Kishk Sa'eedi) and Group 2: Meat and Fish products (Kizota, Lanhouin, Kong), Group 3: Plant based extract (adansonia digitata, hibiscus sabdariffa, ziziphus mauritana).

## 2 References

NF EN ISO 6888-1 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) – Part 1: Technique using Baird-Parker agar.

NOTE: All the procedure is described in the NF EN ISO 6888-1. We only focus on the item 4 Principle and described some important points at the item 9 Critical points or note of the procedure.

## 3 Definitions

## 4 Principle

- 5 *The surface of a selective agar, in two series of Petri dishes, is inoculated with a defined amount of liquid sample, or of the primary suspension if it is not a liquid product.*
- 6 *At the same conditions, the other decimal dilutions obtained from a liquid sample or primary suspension are inoculated into two plates per dilution. The plates are incubated under aerobiose at 37 °C and examined at 24 h and 48 h.*
- 7 *The number of coagulase-positive staphylococci per milliliter or gram of sample is calculated after counting the typical and/or atypical colonies from selected plate (s) given significant result, and confirmed by a positive coagulase test.*

## 5 Reagents

## 6 Apparatus

## 7 Procedure

## 8 Expression of results

### 9 Method of calculation and formulae

#### 9.1.1 Calculation

#### 9.1.2 Formulae

### 10 Repeatability

## 9 Critical points or Note on the procedure

Refer to the items:

### 5 Reagents

Commercial complete dehydrated media and reagents can be used, in this case follow recommendation of the producer.

The sulfamethazine solution should be added to the Baird Parker agar only when the presence of *Proteus* is expected in the sample.

### 7 Procedure

#### - PLATE SELECTION AND INTERPRETATION:

- NOTE 1 Typical colonies on Baird Parker medium are black to gray, brilliant and convex (1 mm to 1.5 mm in diameter after 24 h of incubation, and 1.5 to 2.5 mm after 48 h of incubation), and surrounded by a clear zone which can be partially opaque.
- NOTE 2 Atypical colonies have the same diameter of typical colonies and can present one of the morphologies:
  - a) Black and brilliant colonies with or without a thin white board; without clear or opaque zone;
  - b) Gray colonies without clear zone.

Atypical colonies are occasionally formed by coagulase-positive staphylococci contaminating, for example, dairy products, shrimp and offal. They are less frequent for coagulase-positive staphylococci found in other products.

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SOPs for microbiological methods for Group 1, 2 and 3**

*S. aureus* strains isolated from frozen or desiccated foods that have been stored for extended periods frequently develop less black coloration than typical colonies and may have rough appearance and dry texture.

- NOTE 2 : Bacteria belonging to other genera can form colonies similar to those of staphylococci on Baird Parker agar. Performing Gram staining and the test for catalase before confirmation allow differentiating them. Staphylococci are cocci Gram-positive and catalase positive.

## **10 Test report**

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification of the sample.

## **11 Revision record**

<b>Date</b>	<b>Responsible person</b>	<b>Description of change</b>
09/26/11	Meeting group	

**G. Procedure for *Listeria monocytogenes* detection**  
(Group 1 and 2)

<b>Procedure for <i>Listeria monocytogenes</i> detection</b>		
SOP Number: <b>Micro-06</b>		
Date of creation: 08/07/2010	Date of revision:09/26/11	
Written by : Edna Froeder Arcuri		
For information on this SOP please contact : Edna Froeder Arcuri		
<b>This document has been approved by :</b>		
Partner	Name of the person who approved	Date
		09/26/11
CIRAD	Didier Montet	
UAC	Victor Anihouvi	
UCAD	Nicolas Ayessou	
FAAU	Sameh Awad	
ESB	Manuella Pintado	
FRI	Wisdom Amoa	



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## 1 Scope and application

This procedure can be applied to Group 1: Cereal based products (Akpan, Kenkey, Gowé, Kishk Sa'eedi) and Group 2: Meat and Fish products (Kizota, Lanhouin, Kong).

## 2 References

NF EN ISO 11290-1

Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes* – Part 1: Detection method

Norm ISO 11290-1/ A1:2004

Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes* – Part 1: Detection method

Amendment 1: Modification of the isolation media, of the haemolysis test and inclusion of precision data.

NOTE: All the procedure is described in the NF EN ISO 11290-1 and ISO 11290-1/ A1:2004 . We only focus on the item 4 Principle and described some important points at the item 9 Critical points or note of the procedure.

## 3 Definitions

## 4 Principle

For the detection of *Listeria monocytogenes* it is necessary four successive phases:

### **4.1 Primary enrichment in a selective enrichment broth with reduced concentration of selective agents (Haft-Fraser broth)**

The primary enrichment broth, containing one volume of lithium chloride and haft volume of acriflavin and nalidixic acid (Haft-Fraser broth) is inoculated with the food sample.

Incubate at 30 °C for 24 h.

### **4.2 Secondary enrichment in selective enrichment broth with full concentration of selective agents (Fraser broth)**

The complete secondary enrichment broth (Fraser broth) is inoculated with the culture obtained in 4.1.

Incubate at 37 °C for 48 h.

### **4.3 Isolation and identification**

The two selective agar are inoculated with the cultures obtained in 4.1 and 4.2,;

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- Oxford Agar;
- Agar Listeria according to Ottaviani and Agostini (ALOA)

The agar plates are incubated at 37 °C and examined at 24 h, if necessary at 48 h for presence of colonies characteristic that may be *Listeria monocytogenes*.

#### **4.4 Confirmation**

The presumptive colonies isolated in 4.3 are isolated and confirmed with appropriate morphological, biochemical and physiological tests.

### **5 Reagents**

### **6 Apparatus**

### **7 Procedure**

### **8 Expression of results**

#### **8.1 Method of calculation and formulae**

##### **8.1.1 Calculation**

##### **8.1.2 Formulae**

#### **8.2 Repeatability**

### **9 Critical points or Note on the procedure**

Refer to the items:

#### **4 Principle**

**Note** – *Listeria* spp. can be present in low numbers and usually they are accompanied by a great number of microorganisms from other genera; therefore, a selective enrichment phase is necessary. The primary selective enrichment broth with reduced concentration of selective agents may allow recuperation of injured *Listeria* spp.

#### **7 Procedure**

- **SAMPLE PREPARATION AND DILUTION :**
- For preparation of the primary dilution : aseptically weigh *X* g of product into 9*X* ml of Haft-Fraser broth to get dilution 1:10
- **ISOLATION AND IDENTIFICATION :**

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SOPs for microbiological methods for Group 1, 2 and 3**

- Typical colonies of *Listeria* spp. on Oxford agar at 24 h of incubation are small (1 mm) and of gray/black color with black halo. After 48 h, the colonies are black with a black halo.
- Typical colonies of *L. monocytogenes* on ALOA are green-blue and have a zone of lipolysis around them (opaque halo).
  - Obs.: some strains of *L. monocytogenes* present pale halo (or even no halo at all in case of stress, especially of acid stress).

## **10 Test report**

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification for the sample.

## **11 Revision record**

<b>Date</b>	<b>Responsible person</b>	<b>Description of change</b>
09/26/11	Meeting group	

**H. Procedure for *Salmonella* spp. detection**  
(Group 1 and 2)

<b>Procedure for <i>Salmonella</i> spp. detection</b>		
SOP Number: <b>Micro-07</b>		
Date of creation: 08/26/10	Date of revision: 09/26/11	
Written by : Edna Froeder Arcuri		
For information on this SOP please contact : Edna Froeder Arcuri		
<b>This document has been approved by :</b>		
Partner	Name of the person who approved	Date
		09/26/11
CIRAD	Didier Montet	
UAC	Victor Anihouvi	
UCAD	Nicolas Ayessou	
FAAU	Sameh Awad	
ESB	Manuella Pintado	
FRI	Wisdom Amoa	

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## 1 Scope and application

This procedure can be applied to Group 1: Cereal based products (Akpan, Kenkey, Gowé, Kishk Sa'eedi) and Group 2: Meat and Fish products (Kizota, Lanhouin, Kong).

## 2 References

International Standard – ISO 6579: 2002 Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.

NOTE: All the procedure is described in the ISO 6579:2002. We only focus on the item 4 Principle and described some important points at the item 9 Critical points or note of the procedure.

## 3 Definitions

## 4 Principle

For the detection of *Salmonella* it is necessary four successive phases:

### 4.1 Pre enrichment in non selective broth

A defined amount of food sample is added to Buffered Peptone Water (BPW) at ambient temperature, mixed and then incubate at  $37\text{ °C} \pm 1\text{ °C}$  for  $18\text{ h} \pm 2\text{ h}$ .

### 4.2 Enrichment in selective broth

The Rappaport Vassiliadis broth with soya (RVS broth) and the Muller Kauffmann tetrathionate-novobiocin broth (MKTTn) are inoculated with the culture from 4.1.

### 4.3 Isolation and identification

Inoculation, from the cultures obtained in 4.2, the surface of the two selective agar:

- Xylose-Lysine-Desoxycholate agar (XLD agar) ;
- One other selective agar, of choice, complementary to XLD agar, that allow growth of *Salmonella* lactose positive, including *Salmonella* Typhi and *Salmonella* Paratyphi.

The XLD agar plates are incubated at  $37\text{ °C} \pm 1\text{ °C}$  for  $24\text{ h} \pm 3\text{ h}$ . For the second medium follow manufacturer instructions.

#### **4.4 Confirmation**

Streak the presumptive colonies isolated in 4.3, and confirm them with appropriate biochemical and serological tests.

### **5 Reagents**

### **6 Apparatus**

### **7 Procedure**

### **8 Expression of results**

#### **8.1 Method of calculation and formulae**

##### **8.1.1 Calculation**

##### **8.1.2 Formulae**

#### **8.2 Repeatability**

### **9 Critical points or Note on the procedure**

Refer to the item:

#### **7 Procedure**

##### **- SAMPLE PREPARATION AND DILUTION :**

- Each sample should be mixed to ensure homogeneity before aseptically withdrawing a 25 g analytical unit. Samples should be pre-enriched at a 1:9 sample-to-broth ratio:
  - aseptically weigh 25 g of product into 225 ml of buffered peptone water (BPW).
- If the amount of product is not 25 g, use the quantity of BPW to obtain dilution 1/10.
- For acid or acidifying foods, take care that the pH do not go below 4,5 during pre enrichment. The pH of these foods is more stable when using double strength BPW.

##### **- ISOLATION AND IDENTIFICATION :**

- Typical colonies of *Salmonella* on XLD-agar are Pink colonies with or without black centers. Many cultures of *Salmonella* may produce colonies



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with large, glossy black centers or may appear as almost completely black colonies.

- *Salmonella* H<sub>2</sub>S negative variants (e.g. *Salmonella* Paratyphi A) on XLD agar produce pink colonies with a darker pink center. Lactose-positive *Salmonella* produce yellow colonies without blackening.
- Incubate the second selective agar at adequate temperature and time and then look for characteristic *Salmonella* colonies.

## **10 Test report**

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification for the sample.

## **11 Revision record**

<b>Date</b>	<b>Responsible person</b>	<b>Description of change</b>
09/26/11	Meeting group	

**I. Procedure for *Clostridium perfringens***  
(Group 1, 2 and 3)

<b>Procedure for <i>Clostridium perfringens</i></b>		
SOP Number: <b>Micro-08</b>		
Date of creation: 08/26/10	Date of revision: 09/26/11	
Written by : Edna Froeder Arcuri		
For information on this SOP please contact : Edna Froeder Arcuri		
<b>This document has been approved by :</b>		
Partner	Name of the person who approved	Date
		09/26/11
CIRAD	Didier Montet	
UAC	Victor Anihouvi	
UCAD	Nicolas Ayessou	
FAAU	Sameh Awad	
ESB	Manuella Pintado	
FRI	Wisdom Amoa	

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## 1 Scope and application

This procedure can be applied to Group 1: Cereal based products (Akpan, Kenkey, Gowé, Kishk Sa'eedi) and Group 2: Meat and Fish products (Kizota, Lanhouin, Kong), Group 3: Plant based extract (adansonia digitata, hibiscus sabdariffa, ziziphus mauritana).

## 2 References

NF EN ISO 7937

Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of *Clostridium perfringens* – Colony-count method.

NOTE: All the procedure is described in the NF EN ISO 7937. We only focus on the item 4 Principle and described some important points at the item 9 Critical points or note of the procedure.

## 3 Definitions

## 4 Principle

- *Petri dishes are inoculated with a specified amount of a liquid sample or primary dilution if it is not a liquid product.*
- Others Petri dishes are inoculated, at the same conditions, with decimal dilutions obtained from a liquid sample or primary dilution.
- A selective medium is added (pour plate method) and after solidification, it is covered by a layer of the same medium. The plates are incubated in aerobic conditions at 37 °C for 20 h ± 2 h.
- The characteristic colonies are counted.
- The characteristic colonies are confirmed and the number of *C. perfringens* per millilitre or gram of sample is calculated.

## 5 Reagents

## 6 Apparatus

## 7 Procedure

## 8 Expression of results

### - **Method of calculation and formulae**

#### 8.1.1 Calculation

#### 8.1.2 Formulae

### - **Repeatability**

## 9 Critical points or Note on the procedure

Refer to the item:

### 7 Procedure

#### - **SAMPLE PREPARATION AND DILUTION :**

- To prepare the decimal dilutions use peptone saline solution or buffered peptone water.

#### - **COUNTING AND SELECTING COLONIES :**

- After the incubation period, select plates contain less than 150 colonies, and if possible, plates from two consecutive dilutions for counting.
- Count the characteristic colonies of *C. perfringens* and select five colonies for confirmation.

#### - **BIOCHEMICAL CONFIRMATION:**

- The reaction in lactose sulphite medium (LS) at 46 °C is very specific of *Clostridium perfringens* and some colonies of *Clostridium paraperfringens* and *Clostridium absonum*. It is thus not necessary to make sure that the black colonies on SC agar are pure, before inoculating the thioglycolate broth and then the lactose sulphite medium.
- To use well isolated colonies for confirmation.

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SOPs for microbiological methods for Group 1, 2 and 3**

WARNING - for health reasons, it is desirable to carry out the nitrite test under a hood.

## **10 Test report**

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification for the sample.

## **11 Revision record**

<b>Date</b>	<b>Responsible person</b>	<b>Description of change</b>
09/26/11	Meeting group	

**J. Procedure for enumeration of yeast and moulds – Colony-count at 25 °C**

(Group 1, 2 and 3)

<b>Procedure for enumeration of yeast and moulds – Colony-count at 25 °C</b>		
SOP Number: <b>Micro-09</b>		
Date of creation: 08/25/10	Date of revision: 09/26/11	
Written by : Edna Froeder Arcuri  For information on this SOP please contact : Edna Froeder Arcuri		
<b>This document has been approved by :</b>		
Partner	Name of the person who approved	Date
		09/26/11
CIRAD	Didier Montet	
UAC	Victor Anihouvi	
UCAD	Nicolas Ayessou	
FAAU	Sameh Awad	
ESB	Manuella Pintado	
FRI	Wisdom Amoa	

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**AFTER (G.A n°245025) – Deliverables 1.2.3.4, 1.2.3.5, 1.2.3.6  
SOPs for microbiological methods for Group 1, 2 and 3**

## **1 Scope and application**

This procedure can be applied to Group 1: Cereal based products (Akpan, Kenkey, Gowé, Kishk Sa'eedi) and Group 2: Meat and Fish products (Kizota, Lanhouin, Kong), Group 3: Plant based extract (adansonia digitata or baobab), hibiscus sabdariffa, ziziphus mauritana).

## **2 References**

International Standard – NF ISO 7954, Microbiology – General guidance for enumeration of yeast and moulds – Colony count technique at 25 °C.

NOTE: All the procedure is described in the NF ISO 7954. We only focus on the item 4 Principle and described some important points at the item 9 Critical points or note of the procedure.

## **3 Definitions**

## **4 Principle**

The enumeration of yeast and moulds involves:

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SOPs for microbiological methods for Group 1, 2 and 3**

- 5 *Inoculation and mixing of the sample or dilutions of the sample with a tempered molten culture medium in Petri plates.*
- 6 *Incubation of the plates at 25 °C for 3, 4 or 5 days, in aerobic conditions.*
- 7 *Calculation of the number of yeast and moulds per millilitre or gram of sample from the number of colonies formed in the selected plates.*

**5 Reagents**

**6 Apparatus**

**7 Procedure**

**8 Expression of results**

**9 Method of calculation and formulae**

**9.1.1 Calculation**

**9.1.2 Formulae**

**10 Repeatability**

**9 Critical points or Note on the procedure**

Refer to the items:

**7 Procedure**

- **SAMPLE PREPARATION AND DILUTION :**
- For preparation of decimal dilutions use peptone saline solution (PSD) or buffered peptone water.
- **COLONY COUTING:**
  - o Count the colonies in each plate after 3, 4 and 5 days. After five days of incubation, select plates containing less than 150 colonies. If parts of the plate are overgrown by mould colonies, or if it is difficult to count isolated colonies, consider the counts from day 4 or even day 3 of incubation. In

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this case, the period of incubation (3 or 4 days) must be cited at the Test report.

- If necessary, proceed a microscopic exam for morphological differentiation of colonies of yeast and moulds from bacterial colonies.

**8 Result expression:**

- Record the count as two significant numbers and expressed as a power of 10. When the third number is less than five, do not change the preceding number; when the third number is five or more, increase the preceding number by one unit.
  - Example: 38500 is expressed as  $3,9 \times 10^4$ .

**10 Test report**

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification of the sample.

**11 Revision record**

<b>Date</b>	<b>Responsible person</b>	<b>Description of change</b>
09/26/11	Meeting group	

## **K. Procedure for enumeration of lactic acid bacteria**

(Group 1 and 2)

<b>Procedure for enumeration of lactic acid bacteria</b>		
SOP Number: <b>Micro-10</b>		
Date of creation: 03/05/2011	Date of revision: 09/26/2011	
Written by : Marina Rivollier & Edna Froeder Arcuri For information on this SOP please contact : Marina Rivollier & Edna Froeder Arcuri		
<b>This document has been approved by :</b>		
Partner	Name of the person who approved	Date:09/26/2011
CIRAD	Didier Montet	
UAC	Victor Anihouvi	
UCAD	Nicolas Ayessou	
FAAU	Sameh Awad	
ESB	Manuella Pintado	
FRI	Wisdom Amoa	

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## **1 Objet et domaine d'application**

This procedure can be applied to Group 1: Cereal based products (Akpan, Kenkey, Gowé, Kishk Sa'eedi) and Group 2: Meat and Fish products (Kizota, Lanhouin, Kong).

## **2 Diffusion**

## **3 Documents de référence**

- Document 1002 du COFRAC,
- norme AFNOR NF V 04-503.

## **4 Documents générés**

## **5 Contenu**

### **5.1 Principe**

- ensemencement en profondeur d'un milieu sélectif solide (MRS, pH 5,7) avec une quantité déterminée de la suspension mère ou des dilutions,
- incubation à 30°C pendant 72 h.

### **5.2 Matériel utilisé**

- hotte à flux laminaire (M 190),
- bain-marie (M 135 et M 142),
- étuve à 30°C (M 113),
- compteur de colonies.

### **5.3 Réactifs et consommables**

- pipettes stériles jetables de 1 ml,
- milieu MRS (gélose de Man, Rogosa et Sharpe),
- solution d'acide sorbique à 1,4 % dans une solution de NaOH 1N, stérilisée par filtration,
- filtre de préparation de l'acide sorbique,

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\* Préparation de la solution d'acide sorbique :

- solution mère : dissoudre l'acide sorbique à 14 g qsp 1 l dans une solution de NaOH 1N - stériliser par filtration,
- compléter stérilement le milieu MRS après autoclavage par 10 ml d'acide sorbique qsp 100 ml de milieu.

## **5.4 Protocole**

Ce protocole est suivi par tout(e) technicien(ne) du laboratoire de microbiologie habilité(e) à le réaliser.

### **5.4.1. Ensemencement**

Travailler sous la hotte à flux laminaire, (ou au bec bunsen)

- utiliser une boîte par échantillon et par dilution,
- transférer dans chaque boîte 1 ml de la suspension mère ou de la dilution (utiliser une pipette stérile),  
Si l'échantillon est susceptible d'apporter des levures, compléter le milieu avec de l'acide sorbique de concentration finale 1,4 % (ex : ajouter 100 ml d'acide sorbique qsp 1 l de milieu),
- couler dans chaque boîte environ 15 ml de milieu MRS à 47°C ± 2°C (température du bain-marie),
- homogénéiser soigneusement,
- laisser refroidir les boîtes sur une surface fraîche, la solidification du milieu ne doit pas excéder 10 minutes
- préparer 1 témoin de stérilité en coulant environ 13 ml de milieu dans une boîte vide. L'identifier par :
  - \* témoin Sté,
  - \* la date,
  - \* le nom du milieu.

### **5.4.2. Incubation**

- retourner les boîtes et les incuber 72 h à 30°C.

### **5.4.3. Lecture et expression des résultats**

*Cas général :*

Retenir les boîtes ayant entre 15 et 300 colonies

Appliquer la formule suivante pour obtenir le nombre de bactéries lactiques par gramme de produit :

$$N = \frac{\sum C}{V(n_1 + 0.1n_2)d}$$

$\Sigma C$  : somme des colonies comptées sur les boîtes retenues de deux dilutions successives et dont au moins une contient au minimum 15 colonies

V : volume de l'inoculum appliqué à chaque boîte en ml. Ici V=1ml

$n_1$  : nombre de boîtes retenues à la première dilution. Ici  $n_1=1$

$n_2$  : nombre de boîtes retenues à la deuxième dilution. Ici  $n_2$  peut être égal à 1 ou 0 (si on n'utilise que la troisième dilution)

Si  $n_2=1$ , alors  $N = \frac{\sum C}{1.1d}$

Si  $n_2=0$ , alors  $N = \frac{\sum C}{d}$

*Estimation des petits nombres :*

Si la boîte, au niveau de l'échantillon pour essai (produits liquides) ou de la suspension mère (autres produits) ou de la première dilutionensemencée ou retenue, contient moins de 15 colonies, calculer le nombre estimé NE de bactéries lactiques présentes dans l'échantillon pour essai à l'aide la formule :

$$NE = \frac{C}{V*d}$$

C : nombre de colonies comptées

V : volume de l'inoculum appliqué à la boîte en ml. Ici, V=1

d : taux de dilution de la suspension mère ou de la première dilutionensemencée ou retenue

On obtient donc  $NE = \frac{C}{d}$

*Tolérances :*



Dans le cas où le nombre de colonies comptées est supérieur à 300 pour la boîte d'une première dilution  $d_1$ , avec un nombre de colonies inférieur à 15 pour la boîte de la dilution  $d_2$  suivante :

- si le nombre de colonies, pour la boîte de la dilution  $d_1$ , est compris dans l'intervalle 334 à 300 (partie supérieure de l'intervalle de confiance d'une moyenne pondérée égale à 300), utiliser le mode de calcul du cas général
- si le nombre de colonies, pour la boîte de la dilution  $d_1$ , est supérieur à 334 (limite supérieure de l'intervalle de confiance d'une moyenne pondérée égale à 300), ne retenir que le résultat des comptages de la dilution  $d_2$  et procéder à une estimation des petits nombres, sauf, dans le cas d'un nombre maximum fixé à 300 pour le comptage des colonies, si ce dernier résultat est inférieur à 8 (limite inférieure de l'intervalle de confiance d'une moyenne pondérée égale à 15) car l'écart entre les deux dilutions n'est alors pas acceptable.

*Arrondis :*

Arrondir les résultats à 2 chiffres significatifs. Pour cela, si le troisième chiffre est inférieur à 5, le chiffre précédent n'est pas modifié ; si le troisième chiffre est supérieur ou égal à 5, le chiffre précédent est augmenté d'une unité.

Retenir comme résultat un nombre compris entre 1.1 et 9.9 multiplié par la puissance appropriée de 10, de préférence, ou un nombre entier avec 2 chiffres significatifs.

*Absence de colonie :*

Si on ne lit aucune colonie, indiquer :

- moins de  $1 \times \frac{1}{d}$  par gramme de produit.

d = facteur de dilution

*Traçabilité :*

Indiquer sur le document planification et traçabilité des dénombrements :

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- qui a réalisé chaque étape (initiales),
- les résultats de chaque boîte,
- le résultat du témoin de stérilité.

*Expression finale des résultats :*

Exprimer le résultat sous cette forme :

- "inférieur ou supérieur" si le client demande à se situer par rapport à la réglementation,
- le dénombrement exact quand il est demandé par le client.
- Si le résultat est issu de petits nombre, indiquer qu'il s'agit d'un nombre estimé.