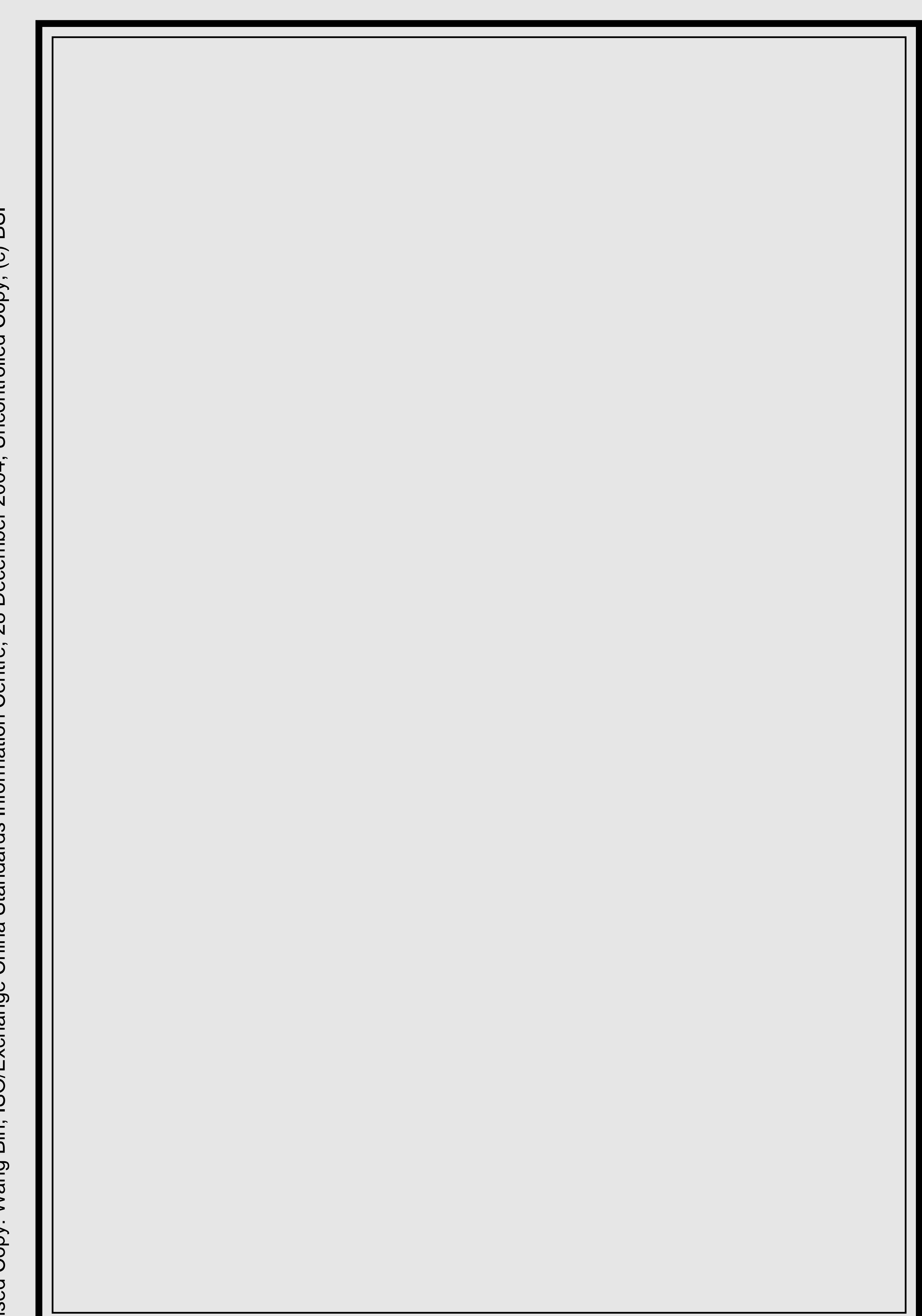

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BRITISH STANDARD

BS EN 14569:2004

Foodstuffs — Microbiological screening for irradiated food using LAL/GNB procedures

The European Standard EN 14569:2004 has the status of a British Standard

ICS 07.100.30

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National foreword

This British Standard is the official English language version of EN 14569:2004.

The UK participation in its preparation was entrusted to Technical Committee AW/-/3, Horizontal analysis, which has the responsibility to:

— aid enquirers to understand the text;

— present to the responsible international/European committee any enquiries on the interpretation, or proposals for change, and keep the UK interests informed;

— monitor related international and European developments and promulgate them in the UK.

A list of organizations represented on this committee can be obtained on request to its secretary.

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This British Standard was published under the authority of the Standards Policy and Strategy Committee on 15 October 2004

Summary of pages

This document comprises a front cover, an inside front cover, the EN title page, pages 2 to 18, an inside back cover and a back cover.

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Amendments issued since publication

Amd. No.	Date	Comments

© BSI 15 October 2004

ISBN 0 580 44604 2

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EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 14569

October 2004

ICS 07.100.30

English version

Foodstuffs - Microbiological screening for irradiated food using LAL/GNB procedures

Produits alimentaires - Dépistages microbiologique des aliments ionisés en utilisant la technique LAL/GNB

Lebensmittel - Mikrobiologisches LAL/GNB-Screeningverfahren zum Nachweis von bestrahlten Lebensmitteln

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Foreword

This document (EN 14569:2004) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by April 2005, and conflicting national standards shall be withdrawn at the latest by April 2005.

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1 Scope

This document specifies a microbiological screening method comprising two procedures, which are carried out in parallel. It permits the identification of an unusual microbiological profile in poultry meat. The presence of a large excess population of dead micro-organisms can under certain circumstances be presumptive of irradiation treatment, which means, that the results of the procedure of the determination of endotoxin concentration in the test sample using the *Limulus* amoebocyte lysate (LAL) test and of the procedure of the enumeration of total Gram negative bacteria (GNB) in the test sample are not radiation specific. Therefore, it is recommended that a positive result be confirmed using a standardized reference method for the detection of irradiated food, e.g. EN 1784, EN 1785 or EN 1786 [1] to [3].

This screening method has been successfully tested by inter-laboratory trials [4], [5], [6] and the procedure is generally applicable to whole or parts of poultry, e.g. breast, legs, wings of fresh, chilled or frozen carcasses with or without skin.

The method can also provide information about the microbiological quality of a product prior to irradiation.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ENV ISO 11133-1, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory (ISO/TS 11133-1:2000).*

ISO 7218, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations.*

3 Principle

The method determines the number of viable Gram negative bacteria present in the test sample and the concentration of bacterial endotoxin present on the surfaces of Gram negative bacteria as lipopolysaccharides (LPS) serving as a measure for the estimation of the amount of total Gram negative bacteria, both viable and dead (Figure C.1). If the difference is high, it is assumed that the sample has been treated by a method of preservation, possibly by treatment with ionising radiation.

4 Procedures

The two procedures that are required to be carried out are:

Procedure 1: Enumeration of total Gram negative bacteria (GNB) in the test sample (according to Annex A)

Procedure 2: Determination of endotoxin concentration in the test sample using the *Limulus* amoebocyte lysate (LAL) test (according to Annex B).

Samples should be analysed immediately upon receipt (by both GNB and LAL) to reduce any further microbial proliferation. If this is not possible, samples should be stored at – 20 °C until examination, which should be carried out as soon as possible.

5 Evaluation

In accordance with the methods specified in Annexes A and B the number of GNB forming units shall be expressed as $\log_{10}$ colony forming unit (cfu) GNB per gram and the concentration of endotoxin expressed as $\log_{10}$ Endotoxin Units (EU) per gram.

The difference between the GNB and the endotoxin results shall be calculated as ($\log_{10}$ EU/g - $\log_{10}$ cfu GNB/g).

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Samples that are identified as having high endotoxin levels but low levels or no detectable GNB show an unusual microbiological profile. Thus, when $\log_{10}$ EU/g - $\log_{10}$ cfu GNB/g is greater than 0 the sample is suspected of having been irradiated and should be subjected to further radiation specific tests.

Samples that are identified as having high endotoxin levels and similarly high Gram negative bacteria counts show a normal microbiological profile. Thus, when $\log_{10}$ EU/g - $\log_{10}$ cfu GNB/g is lower or equal 0, the sample is not suspected of having been irradiated.

Samples that are identified as having endotoxin titres $\log_{10}$ EU/g of lower than 2,0 and low levels of GNB, i.e. $\log_{10}$ cfu GNB/g of lower than 2,0 should be considered inconclusive and should be subjected to further radiation specific tests.

6 Limitations

This method can give only an indication of a possible treatment by ionising radiation. A high amount of dead microorganisms in comparison to the viable fraction can be due to several other reasons. It is therefore necessary to confirm a possible treatment by ionising radiation by a standardized reference method for the detection of irradiated foods. The method is of particular use to routine microbiological laboratories, which may be involved in the examination of foods.

It should be noted that freezing after irradiation can influence the ratio of GNB to EU due to the loss of viability of microorganisms. Conversely, re-growth of bacterial flora can occur in irradiated samples which are stored unfrozen.

7 Validation

The method was developed by the UK Ministry of Agriculture, Fisheries and Food, Food Science Laboratory, Norwich (FSL) and validated by collaborative trial [4], [5], [6]. Twenty UK laboratories participated in the trial (16 Public Analyst Laboratories, 3 Public Health Laboratories and FSL). A commercial poultry processor supplied a single batch of boneless chicken breasts with skin and a single batch of boneless chicken breast fillets. Within 24 h of slaughter, both batches of chicken were transported from the processing plant, under refrigeration conditions (<5 °C), to a commercial irradiation facility. The batch of chicken breasts were subdivided; one third of the batch were retained as control (unirradiated) samples; one third were irradiated at an overall average dose of 2,5 kGy and the remainder irradiated at 5 kGy. The skinless chicken breast fillets were sub-divided into two. Half were retained as controls and the remaining half were irradiated at 2,5 kGy. Samples were irradiated using a Cobalt 60 source. Amber perspex dosimeters[1)] were used to estimate the dose received by measuring spectrometrically a change in absorbance at 530 nm. All chicken pieces were randomly coded and packaged in insulated containers under chill conditions. Samples were then delivered by overnight carrier to arrive at participating laboratories the following morning. Participants were instructed to commence the analysis immediately upon arrival of the samples. Each participant received 10 samples for analysis comprising control samples of chicken breasts with and without skin; chicken breasts with skin irradiated at 2,5 kGy and 5 kGy and skinless breasts irradiated at 2,5 kGy. All samples were dispatched randomly as blind ‘pairs’. Participants were asked to examine each sample only once and report the results obtained from the LAL test and enumeration of GNB.

Using the evaluation criteria detailed in Clause 5, 100 % of all unirradiated samples, 97 % of chicken with skin irradiated at 2,5 kGy, 82 % of those irradiated at 5 kGy and 88 % of the skinless fillets irradiated at 2,5 kGy were correctly identified. All other samples were reported as ‘inconclusive’ due to the very low EU titres obtained.

---

1) Amber perspex dosimeter is an example of a suitable product commercially available from Harwell, UK which was used for the interlaboratory test. This information is given for the convenience of users and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

8 Test report

The test report shall contain at least the following:

a) information necessary for identification of the sample;

b) a reference to this document;

c) date of sampling and sampling procedure (if known);

d) date of receipt and thermal status of the sample when received;

e) date of test;

f) storage conditions of the sample in the laboratory after receipt;

g) the result;

h) any particular points observed in the course of the test;

i) any operations not specified in the method or regarded as optional which might have affected the results.

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Annex A
(normative)

Enumeration of total viable Gram negative bacteria (GNB)

A.1 Principle

The enumeration of GNB which form colonies aerobically at 21 °C ± 1 °C for 24 h ± 1 h on a selective medium containing nisin, penicillin-G and crystal violet is carried out in four successive stages.

— Preparation of initial test suspension in endotoxin-free water;

— Surface inoculation of non-selective nutrient solid agar medium to allow resuscitation of stressed cells; pre-incubation at ambient temperature for 90 min;

— Overlaying of the non-selective medium following the resuscitation period with selective agar medium containing nisin, penicillin-G and crystal violet and incubation at 21 °C ± 1 °C for 24 h ± 1 h;

— Enumeration.

A.2 Diluents, culture media, and reagents

A.2.1 General

For uniformity of results, it is recommended that media be prepared from dehydrated commercial formulations where possible. All chemicals used in the preparation of culture media shall be of analytical quality. Water shall be distilled or de-ionised and free from inhibitory substances. Applicable provisions of ISO 7218 shall be respected.

A.2.2 Endotoxin-free water[2)]

A.2.3 Peptone salt solution

Sodium chloride	8,5 g
Enzymatic digest of casein	1,0 g
Water	1 000 ml

Dissolve the constituents in the water. Mix well and if necessary adjust pH so that after sterilisation it is 7,0 ± 0,2 at 25 °C. Sterilise by autoclaving at 121 °C ± 1 °C for 15 min. The diluent may be stored for maximum two weeks at 4 °C ± 1 °C.

A.2.4 Nutrient agar (NA) medium (non-selective)

Enzymatic digest of casein	5,0 g
Sodium chloride	5,0 g
Yeast extract	2,0 g
Meat extract	1,0 g
Agar	9 g to 18 g[a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

2) Endotoxin (pyrogen) free water can generally be obtained from manufacturers supplying the pharmaceutical industry.

Dissolve the constituents in the water by boiling. Adjust the pH so that after sterilization it is 7,4 ± 0,2 at 20 °C to 25 °C. Sterilise by autoclaving at 121 °C ± 1 °C for 15 min. Cool to 45 °C to 50 °C and dispense approximately 10 ml into sterile Petri dishes.

A.2.5 Gram negative selective agar medium

A.2.5.1 Basal medium

Milk agar (e.g. Oxoid CM21)[3]	24,0 g	
Nisin	40,0 mg[a]	(40 000 International Units/litre)
Water	997 ml	
[a] May need to be adjusted according to commercial activity of the nisin standard.		

Dissolve the constituents in the water by boiling. Adjust the pH so that after sterilisation it is 7,1 ± 0,2 at 20 °C to 25 °C. Distribute into final containers. Sterilise at 121 °C ± 1 °C for 15 min. Cool to 45 °C to 50 °C.

A.2.5.2 Crystal violet solution

Crystal violet	10 mg
Water	10 ml

Dissolve the crystal violet in the water. Filter sterilise through 0,22 µm pore size filter. The crystal violet solution may be stored at 4 °C to 6 °C up to two weeks.

A.2.5.3 Penicillin-G solution

Penicillin-G	100 mg[a]	(20 000 International Units/litre)
Water	10 ml	
[a] May need to be adjusted according to commercial activity of the penicillin standard.		

Dissolve the penicillin-G in the water. Filter sterilise through 0,22 µm pore size filter. The penicillin-G solution may be stored at 4 °C to 6 °C for no longer than one week.

A.2.5.4 Complete Gram negative selective medium

Penicillin-G solution	1,2 ml[a]
Crystal violet solution	2 ml
Basal medium	997 ml
[a] May need to be adjusted according to commercial activity of the penicillin standard.	

Add the crystal violet and penicillin-G solutions aseptically to the molten basal medium tempered to 45 °C ± 1 °C. Mix well and dispense as required.

3) Oxoid CM21 is an example of a suitable product commercially available. This information is given for the convenience of users and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

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A.3 Apparatus

A.3.1

Usual microbiological laboratory equipment and in particular:

A.3.2 Endotoxin-free glassware

Endotoxin-free glassware may be achieved by heating at 180 °C ± 2 °C for 4 h. Alternatively, sterile plastic ware which is usually endotoxin-free may be used in preference to glass.

A.3.3 Peristaltic homogenizer and plastic bags

A.3.4 Sterile plastic universal bottles (approximately 30 ml)

A.3.5 Incubator capable of maintaining a temperature of 21 °C ± 1 °C.

A.3.6 Laboratory oven capable of maintaining a temperature of 180 °C ± 2 °C.

A.4 Procedure

A.4.1 Preparation of test sample and initial suspensions

Aseptically remove 4 different portions of skin of about 2,5 g to form a 10 g composite skin sample. For whole carcasses the 2,5 g portions should be taken from the neck, vent and under each wing. For chicken portions take skin samples from different regions. If skinless chicken pieces are examined use 10 g of surface muscle tissue excised aseptically. Place the skin or muscle portions in a plastic bag and add 90 ml of endotoxin-free water (A.2.2). Homogenize in the peristaltic apparatus (A.3.3) for 1 min to 2 min. Aseptically transfer an appropriate volume (e.g. 30 ml) of this initial suspension into a sterile universal bottle (A.3.4) and retain it at 1 °C to 4 °C for the following procedures (1 ml is needed for GNB and 30 µl are needed for LAL). The LAL procedure should be carried out within 2 h. This initial suspension is equivalent to a 10^{-1} dilution of the original sample.

A.4.2 Preparation of dilutions

Ideally, plate counts should be in the range of 15 to 300 colony forming units (cfu) per plate. Prepare further decimal dilutions for plate counts by adding 1 ml of the initial suspension (A.4.1) to 9 ml of peptone salt solution (A.2.3) to make a 10^{-2} dilution and repeat until the sample is sufficiently diluted (usually to at least 10^{-5}). Not more than 15 min should elapse between preparation of dilutions and plating out.

A.4.3 Plating out

Spread duplicate 0,1 ml aliquots over the surface of 2 dried nutrient agar plates (A.2.4). Leave at ambient temperature (20 °C to 25 °C) for 90 min, then overlay with approximately 10 ml of GNB selective agar (A.2.5.4) at a temperature of 45 °C ± 1 °C. Care should be taken to avoid pouring an overlay which is too thick. Allow to set and incubate in an inverted position at 21 °C ± 1 °C for 24 h ± 1 h.

A.4.4 Enumeration of GNB/g

Count the colonies in each dish containing less than 300 colonies. For a result to be valid, it is considered that it is necessary to count the colonies on at least one dish containing, as a minimum, 15 colonies.

Calculate the number N of GNB present in the test sample, as the weighted mean from 2 successive dilutions using equation (A.1).

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$$N = \frac{\sum c}{V\,[n_1 + (0{,}1 \times n_2)\,d]} \qquad \text{(A.1)}$$

where

$\sum c$ is the sum of the colonies counted on all the dishes retained from 2 successive dilutions and where at least one contains a minimum of 15 colonies;

V is the volume of inoculum applied to each dish, in millilitres (here 0,1 ml);

n_1 is the number of dishes retained at the first dilution;

n_2 is the number of dishes retained at the second dilution;

d is the dilution factor corresponding to the first dilution retained.

EXAMPLE

Dilution	No of colonies per duplicate dish	
10^{-3}	271	240
10^{-4}	30	32

$$N = \frac{573}{0{,}1\,(2 + 0{,}2)\,10^{-3}} = 2{,}6 \times 10^{6}\,\text{cfu GNB}/g$$

A.5 Expression of results

Record the number of cfu GNB/g of skin or muscle as calculated in A.4.4. Express the results as $\log_{10}$ cfu GNB/g. If fewer than 15 colonies are present at the lowest dilution of 10^{-1} report the result as an estimated count (E). If no colonies of GNB are present from the lowest dilution of 10^{-1}, report the result as "GNB not detected".

A.6 Quality control procedures

All culture media shall be tested using appropriate positive and negative control reference cultures using procedures such as those recommended by ENV ISO 11133-1, *Microbiology of food and animal feeding stuffs - Guidelines on preparation and production of culture media - Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory (ISO/TS 11133-1:2000)*.

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Annex B
(normative)

Determination of endotoxin concentration using the *Limulus* amoebocyte lysate (LAL) test

B.1 Principle

The *Limulus* amoebocyte lysate (LAL)-test is a semi-quantitative assay using microtitre plates containing lyophilised amoebocytes from the horseshoe crab which react to form a gel in the presence of endotoxin (also known as lipopolysaccharide) from the cell walls of viable and non-viable Gram-negative bacteria [7].

The determination of the concentration of endotoxin present in the test sample is carried out in 3 successive stages:

— Preparation of dilutions in LAL microtitre plates using the initial suspension of test sample prepared in A.4.1.

— Incubation of LAL microtitre plates at 37 °C for 1 h.

— Visualising gel clots and calculation of endotoxin concentration.

B.2 Reagents

B.2.1 Endotoxin-free water[4)] (see A.2.2)

B.2.2 Lyophilised reference standard[5)] approximately 500 Endotoxin Units (EU)

B.2.3 Toluidine blue dye

Polysorbat 20 (e.g.Tween 20®)[6)]	1,0 g
Toluidine blue	0,2 g
Water	100 ml

Dissolve the constituents in the water and mix thoroughly.

---

4) Endotoxin (pyrogen) free water can generally be obtained from manufacturers supplying the pharmaceutical industry.

5) LPS reference standard is an example of a suitable product available commercially. This information is given for the convenience of users and does not constitute an endorsement by CEN of this product. Alternative methods for the determination of LPS concentration are available and may be substituted provided they have been demonstrated to give equivalent results to the microtitre assay.

6) Tween 20® is an example of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of this product.

B.3 Apparatus

B.3.1

Usual microbiological laboratory equipment and in particular:

B.3.2 Non-circulating water bath

Static water bath capable of maintaining a temperature of 37 °C ± 0,5 °C.

B.3.3 LAL test microtitre plates[7)] (With a sensitivity of 1 EU to 2 EU)

The plates used are standard 96 well plates (12 columns x 8 rows) with alternate columns of empty dilution wells and lysate adsorbed test wells (Figure C.2). The microtitre plates shall be handled with care as excessive vibration can disturb the adsorbed lysate and cause false negative results.

B.3.4 Automatic pipettes capable of dispensing 65 µl and 30 µl volumes.

B.3.5 Parafilm® [7)]

B.3.6 Suction pump (water or mechanical)

B.3.7 Rotary homogenizer

B.4 Procedure

B.4.1 General

The initial suspension prepared in A.4.1 is used for the measurement of endotoxin concentration.

B.4.2 Dilution of sample in the LAL test microtitre plate (see Figure C.3).

Add 65 µl (B.3.4) of endotoxin-free water (A.2.2) to each dilution well of the microtitre plates (B.3.3). Add 30 µl from the initial test suspension (A.4.1) to the first dilution well containing 65 µl of endotoxin-free water (this yields a $10^{-0,5}$ dilution of the initial suspension) and mix the well contents thoroughly by filling and emptying pipette tip taking care not to introduce air bubbles into the microtitre well.

Using a fresh pipette tip, transfer 30 µl from the first dilution well to the first test well (well has lysate adsorbed to base) and another 30 µl from the first dilution well to the second dilution well (this yields a $10^{-1,0}$ dilution of the initial suspension). Using a clean pipette tip, mix thoroughly the contents of the second dilution well.

Transfer 30 µl from the second dilution well to the second test well and another 30 µl to the third dilution well (this yields a $10^{-1,5}$ dilution of the initial suspension). Using a clean pipette tip, mix thoroughly.

Continue with dilution of the test material in the microtitre plate as above until all eight vertical test wells have been filled for each sample.

Four samples may be tested per plate plus controls (B.4.3).

---

7) LAL microtitre plates and Parafilm® are examples of suitable products commercially available. This information is given for the convenience of users and does not constitute an endorsement by CEN of these products.

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B.4.3 Positive and negative controls

B.4.3.1 General

In addition to the test samples, positive and negative controls shall also be incorporated onto each plate.

B.4.3.2 Positive control

Resuspend the lyophilised reference standard (B.2.2) in endotoxin-free water to prepare a 50 EU/ml solution. Homogenize for 2 min. Test as for test samples as described in B.4.2.

B.4.3.3 Negative control

Add 30 µl endotoxin-free water (B.2.1) to two spare test wells in the microtitre plate.

B.4.4 Incubation of the LAL test

Cover the microtitre plate with Parafilm® (B.3.5) immediately after preparation and float on a water bath at 37 °C ± 0,5 °C for 1 h.

B.4.5 Visualisation of gel clots

After the incubation period, remove the plate from the water bath and carefully blot dry. Remove the Parafilm® and add 1 drop of toluidine blue dye (B.2.3) to each test well. In order to detect gel formation, aspirate each test well with the aid of a Pasteur pipette fitted to a gentle suction pump (B.3.6). Gently touch the bottom of the well with the tip of the Pasteur pipette with the suction on (care should be taken not to scrape the Pasteur pipette across the bottom of the well since this will disrupt any gel clot that has formed so that it may be sucked out accidentally). Record a positive reaction (+) where a gel has formed in the well (when a hole in the formed gel where the Pasteur pipette touched will be visible). Record a negative reaction (-) if the contents of the well are completely sucked out. Record a partial reaction (+/-) when there is evidence of slight gel formation in the base of the well, i.e. well is not completely empty.

B.5 Calculation of endotoxin content of test sample

The endotoxin content of the test sample is reported as EU per gram of sample. Determine the titre of the last positive well in the sequences of dilutions.

Examples to determine the titre:

EXAMPLE 1:

Test well	LAL reaction	Dilution
1	+	$10^{-0,5}$
2	+	$10^{-1,0}$
3	+	$10^{-1,5}$
4	-	$10^{-2,0}$
Titre = 1,5		

EXAMPLE 2:

Test well	LAL reaction	Dilution
1	+	$10^{-0.5}$
2	+	$10^{-1,0}$
3	+/- i. e partial gel	$10^{-1,5}$
4	-	$10^{-2,0}$
5	-	$10^{-2,5}$
Titre = 1,25		

Calculate the number of EU per gram of sample using equations (B.1) and (B.2):

$$\text{EU/ml} = 10^{(\text{titre})} \times \text{sensitivity of the lysate (given by manufacturer on the microtitre plate)} \quad (B.1)$$

$$\text{EU/g} \triangleq \text{EU/ml} \times 10 \quad (B.2)$$

B.6 Expression of results

Express the result as $\log_{10}$ EU/g.

B.7 Quality control procedures

Positive (B.4.3.2) and negative (B.4.3.3) endotoxin controls shall be included on each microtitre plate. If the negative control shows positive, all tests shall be repeated with a new batch of endotoxin-free water. If the positive control differs by more than 0,25 titre of the expected reference titre as stated by the manufacturer (B.2.2), the samples shall be re-tested using another microtitre plate.

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Annex C
(informative)

Figures

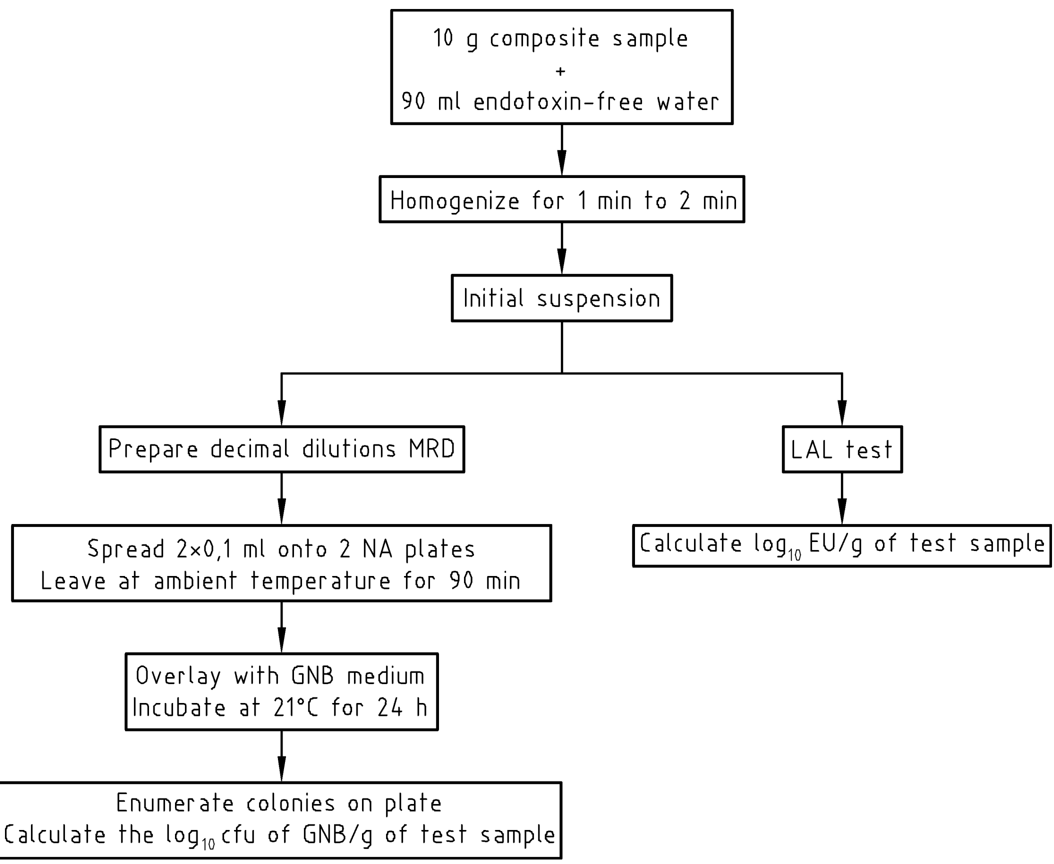

Figure C.1 — Schematic representation of LAL/GNB method

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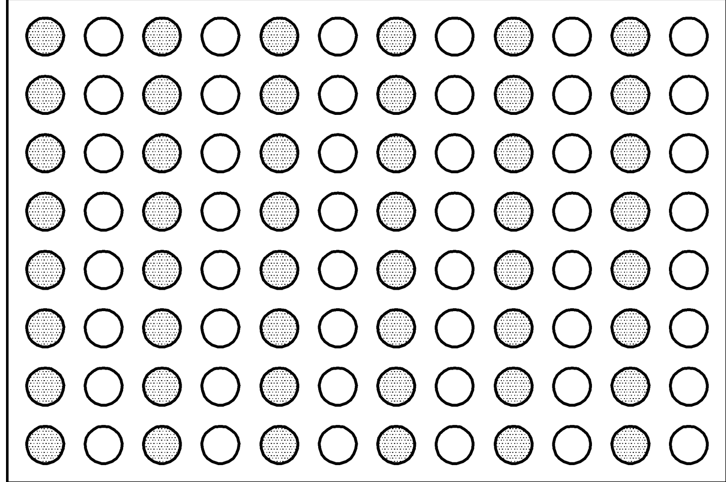

Figure C.2 — Schematic representation of a complete LAL microtitre plate[8] (see B.3.3)

Dilution Well

Lysate Test Well

[8] LAL microtitre plates is an example of a suitable product commercially available. This information is given for the convenience of users and does not constitute an endorsement by CEN of this product.

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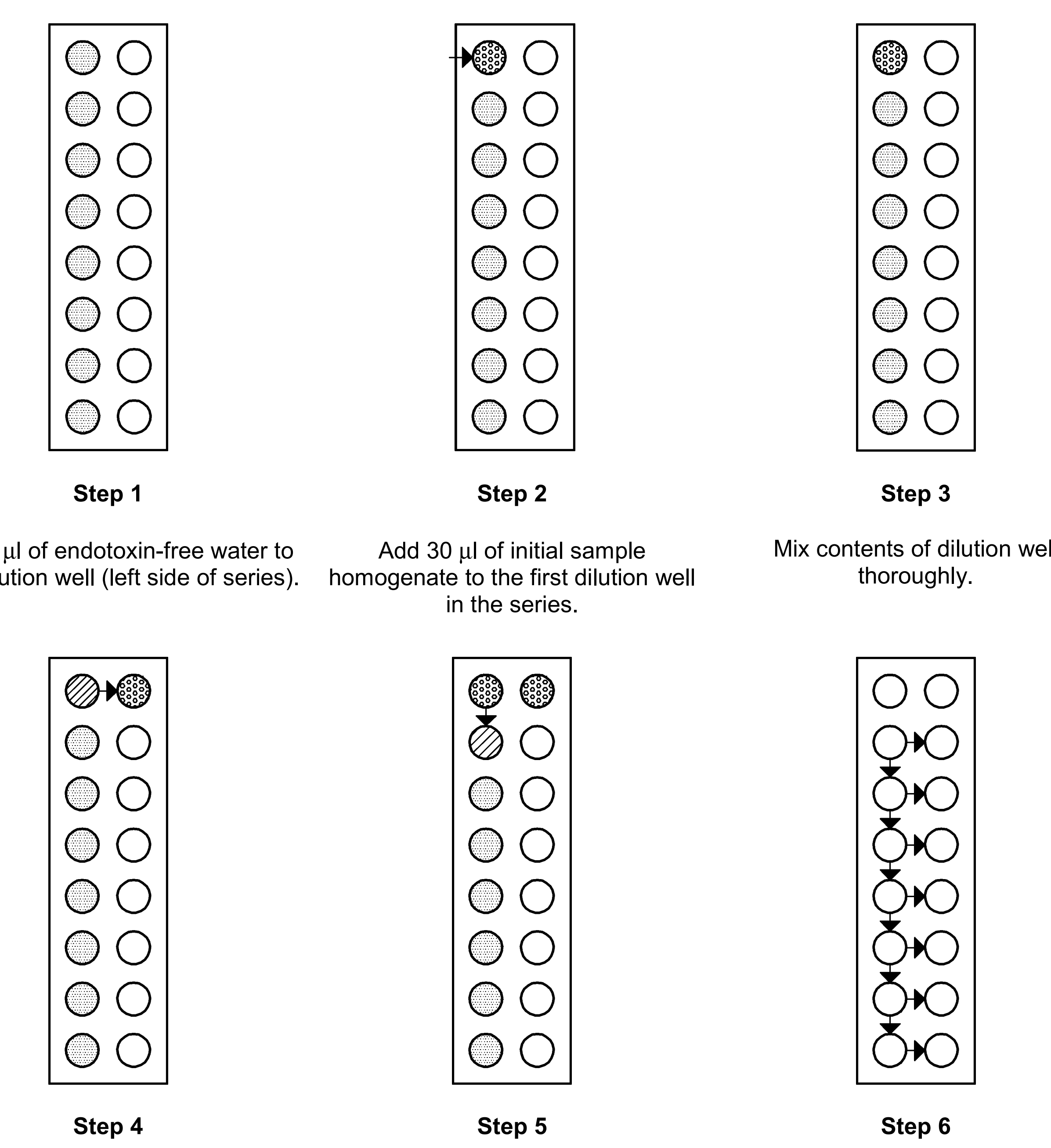

Figure C.3 — Schematic representation of dilution and test wells series in a LAL 96 well microtitre plate[9] (see B.4.2)

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