

FOR COMMENT ONLY

PROPOSAL FOR A

TRINIDAD AND TOBAGO STANDARD

COMPULSORY

HAND DISHWASHING DETERGENT – SPECIFICATION

**PCTTS 575:20XX
(1st Revision)**

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Public Comment

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The Bureau develops standards through consultation with relevant interest groups, and public comment is invited on all draft standards before they are declared as Trinidad and Tobago Standards in accordance with the provisions of the Standards Act.

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NOTE: In order to keep abreast of progress in the industries concerned, Trinidad and Tobago Standards are subject to periodic review. Suggestions for improvements are welcomed.

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Committee

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Public Comment

Foreword

This specification was declared a Trinidad and Tobago Standard on, after the draft finalized by the Specifications Committee for Hand Dishwashing Detergent had been approved by the Trinidad and Tobago Bureau of Standards. This standard is a revision of TTS 575: 2000, *Hand dishwashing detergent – Specifications*.

This standard was revised to reflect the new format of Trinidad and Tobago standards and to address the issues listed below:

- a) recent changes in technology, in particular, the inclusion of a test method for the quantification of amphoteric surfactants;
- b) environmental concerns and regulations;
- c) the modification of the scope to include detergent in any concentration and form; and
- d) the revision of the sampling method.

The Trinidad and Tobago Bureau of Standards has recommended that this standard be declared a compulsory standard to protect the consumer or user against danger to health or safety.

This revision will become effective as a compulsory standard on a date to be notified by the Minister responsible for trade and industrial development in a notice to be published in the Trinidad and Tobago Gazette, as required by the Standards Act.

This national standard was developed for compliance by manufacturers, importers and distributors of hand dishwashing detergents.

In formulating this standard considerable assistance was derived from the following standard which was still current when this standard was being developed:

Malaysian Standard

MS 791: 2001, *Specification for liquid detergent for household hand dish washing (first revision)*

Annexes A, B, C, D, E and F form a normative part of this standard.

Annex A outlines the procedure to determine rinsing properties.

Annex B outlines the procedure to determine the type of surface active agent present.

Annex C provides guidance for the preparation of dilutions for microbial examination.

Annex D provides guidance for the enumeration of coliforms.

Annex E provides guidance for the enumeration of yeasts and moulds.

Annex F outlines the procedure to determine the presence of Gram-negative bacteria.

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1 Scope

This national standard specifies the chemical and physical requirements for detergents used for hand dishwashing. It also specifies requirements for labelling, packaging, sampling, and test methods.

This national standard covers hand dishwashing detergent in any commonly available form.

NOTE Commonly available forms include liquid, gel, paste, bar, and powder.

This national standard does not specify requirements for any detergents formulated for use in automatic dishwashers.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this national standard.

ASTM International

ASTM D 1193-06, *Standard specification for reagent water*

ASTM D 2667-95 (Reapproved 2008), *Standard test method for biodegradability of alkylbenzene sulfonates*

British Standards Institution

BS 3762:Section 3.7:1986, *Analysis of formulated detergents – Part 3: Quantitative test methods – Section 3.7: Method for determination of total non-ionic matter content*

BS EN 13270:2001, *Surface active agents – Determination of the active matter content in alkyldimethylbetaines*

International Organization for Standardization

ISO 2271:1989, *Surface active agents – Detergents – Determination of anionic-active matter by manual or mechanical direct two-phase titration procedure*

ISO 2871-1:1988, *Surface active agents – Detergents – Determination of cationic-active matter content – Part 1 – High-molecular-mass cationic-active matter*

ISO 2871-2:1988, *Surface active agents – Detergents – Determination of cationic-active matter content – Part 2: Cationic-active matter of low molecular mass (between 200 and 500)*

Trinidad and Tobago Bureau of Standards

TTS 76: Part 15, *Requirements for labelling - Labelling of general household and automotive cleaning chemicals*

3 Terms and definitions

For the purpose of this national standard the following terms and definitions shall apply.

3.1

address

the full postal address of the principal place of business or registered office of the manufacturer or distributor of the product

3.2

amphoteric surface active agent

a surface active agent that is capable of forming, in aqueous solution, either surface active anions or surface active cations depending on the pH

3.3

anionic surface active agent

a surface active agent which ionizes in aqueous solution to produce negatively charged organic ions which are responsible for the surface activity

3.4

batch

the material from a single mix or, in the case of a continuous production process, the material from a single day's production

3.5

biodegradable

capable of being decomposed into innocuous products by living matter

3.6

cationic surface active agent

a surface active agent which ionizes in aqueous solution to produce positively charged organic ions which are responsible for the surface activity

3.7

coliforms

bacteria which, at the temperature of 37 °C form characteristic colonies in crystal violet neutral red bile lactose agar

NOTE See conditions specified in Annex D.

3.8

detergent

a product specially formulated to dislodge soil from a substrate and keep the soil in a state of solution or dispersion

NOTE Within the context of this national standard, detergent only refers to household hand dishwashing detergent.

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3.9

diluent

an inert substance added to a mixture especially for reducing the concentration of active ingredients

3.10

functional group

an arrangement of atoms within a substance, which confer special properties to the substance

3.11

further decimal dilution

the suspension or solution obtained by mixing a determined amount of the initial suspension with a specified volume of diluent, and by repeating this operation with every dilution thus prepared, until a decimal dilution series, suitable for the inoculation of culture media, is obtained

3.12

homogenous

having a uniform structure and composition

3.13

initial suspension

the suspension, solution or emulsion obtained after a weighed or measured quantity of the product under examination (or of a test sample prepared from the product) has been mixed, with a specified volume of diluent

3.14

lot

the quantity of a detergent that bears the same batch identification, from one manufacturer, submitted at any one time for inspection and testing

3.15

micro-organism

any organism not visible to the naked eye

3.16

non-ionic surface active agent

a surface active agent which does not produce ions in an aqueous solution, but has functional groups which are responsible for its surface activity

3.17

opacifier

a chemical agent added to a material to make the resulting system opaque

3.18

pH

the decimal logarithm of hydrogen ion concentration in moles per litre, which gives a measure of the acidity or alkalinity of a solution

3.19

primary package

the receptacle, container, wrapper, box, confining band or card, in or on which goods are sold, but does not include package liners, shipping containers or any other wrapping or box not customarily displayed to the consumer or purchaser at the point of retail sale

3.20

secondary package

any container intended to protect goods during transport, which is not customarily used to store detergent when displayed for sale

3.21

surface active agent

surfactant

a chemical compound that modifies the physical (mechanical or electrical) properties of a surface or an interface and reduces its surface tension

3.22

yeasts and moulds

micro-organisms which at 25 °C form colonies in a selective medium

NOTE See method specified in Annex E.

4 Requirements

4.1 General

4.1.1 The detergent shall be homogenous and free from foreign matter.

4.1.2 The detergent may contain additives.

EXAMPLE These may include colouring agents, perfumes, opacifiers, viscosity controlling agents, foam control agents, solubilizers and preservatives.

4.1.3 Raw materials used for detergent manufacture shall not cause any harm or threat to human health or the environment, when used in accordance with the stipulated directions.

4.1.4 The manufacturer shall be able to provide evidence in support of clause 4.1.3, in a timely manner to the appropriate authority.

4.1.5 The manufacturer, importer and distributor of the finished product shall be able to provide evidence that the product, when used in accordance with the stipulated directions, shall not cause any harm or threat to human health or the environment.

NOTE While it is recognized that some persons may display an allergic reaction to common ingredients of detergents, it is not intended that this clause should cover the requirements of such allergic reactions.

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4.1.6 The detergent shall be readily soluble in water.

4.1.7 The detergent, whether diluted or undiluted, shall not have an objectionable odour symptomatic of the degradation of the product.

4.1.8 The detergent shall not leave any residual taste or odour on washed articles.

4.1.9 The detergent shall be free-rinsing.

NOTE See clause 8.1.

4.1.10 The detergents shall be capable of meeting the requirements of this standard after storage for a period of one year in its original sealed container under conditions of temperature of 22 °C – 35 °C.

NOTE This pertains to the physical characteristics of the finished product.

4.2 Specific

4.2.1 The pH value of a solution of detergent of a volume fraction of 1.0 % in water at 25 °C shall be between 6.0 and 8.5. When reporting the value, it shall be expressed to one decimal point.

4.2.2 The minimum percentage of total surface active agents in the detergent shall not be less than 12 % by mass. The surface active agents shall be anionic, cationic, non-ionic, amphoteric or a blend of non-ionic and/or amphoteric, and either cationic or anionic.

4.2.3 The detergent shall contain less than 300 colony forming units (cfu) of coliforms per gram of detergent, less than 10 cfu of yeasts and moulds per gram of detergent and no gram negative bacteria.

4.2.4 The detergent shall be at least 90 % biodegradable.

NOTE See clause 8.7.

5 Package

5.1 There shall be no reaction between the primary package and the detergent which will impair the form or function of the detergent, produce products which may adversely affect human health or the environment, or cause loss of the retentive properties of the package.

5.2 The primary package shall close securely to prevent drying out and contamination of the product.

5.3 The primary package shall be sufficiently strong to prevent leakage arising from the ordinary risks of transport or use.

6 Labelling

6.1 Every individual primary package of hand dishwashing detergent and collective units of such individual primary packages, that are displayed for sale or offered for sale, shall bear a label, which shall state the following information in the English language and in a legible form.

6.1.1 On the main panel of the label, the following information shall be stated:

a) the common name of the product;

EXAMPLE The common name may be "hand dishwashing detergent".

b) the brand name or trade name of the detergent;

c) the statement "KEEP OUT OF THE REACH OF CHILDREN" or other similar statement, which shall be conspicuous; and

d) a statement of the net contents of the package in terms of SI units of mass (weight) or volume;

6.1.2 On the label of each primary package of hand dishwashing detergent offered for sale, the following additional information shall be stated:

a) the name and address of the manufacturer and/or distributor of the detergent;

NOTE The address required above shall be a physical address and not an internet address.

b) the country of origin;

c) the statement "READ INSTRUCTIONS BEFORE USE" or other similar statement;

d) adequate directions as to the manner of the use of the detergent;

e) the main intended uses of the detergent;

NOTE For detergents that claim to be antibacterial or antimicrobial when used as a hand wash, evidence shall be provided to the appropriate authority to support the relevant claim.

f) the common names of all active ingredients;

g) where the packaging is not transparent, the physical form of the detergent;

h) first aid instructions

i) a date indicating the "best before" use period (or other similar designation) from the date of manufacture; and

j) the batch number or lot number stated in code or otherwise.

6.2 In addition to the requirements listed above, the labelling of primary and secondary packages shall comply with TTS 76: Part 15, *Requirements for labelling – Labelling of general household and automotive cleaning chemicals*.

7 Sampling

7.1 Sample Size

7.1.1 Each lot shall be tested separately for compliance to the requirements of this standard. The number of primary packages of detergent to be selected, n , to constitute a representative sample of the lot shall depend on the size of the lot, N . Primary packages shall be selected at random from each lot according to Table 1.

Table 1 — Scale of sampling

Lot size, N (# of primary packages)	Sample size, n (# of primary packages)
2 – 25	2
26 – 150	3
151 – 1200	5
1201 – 35000	8
35001 and over	13

NOTE The sample and lot sizes above were determined using Tables 1 and 2-A of ISO 2859-1:1999, *Sampling procedures by attributes - Part 1: Sampling schemes indexed by acceptance quality limit (AQL) for lot-by-lot inspection*. This applies to a single sampling plan for normal inspection and a special inspection level of S-2.

7.1.2 The lot shall consist of secondary packages of the same batch and date of manufacture.

7.2 Selection of sample primary packages

7.2.1 The primary packages will be selected randomly using random number tables or systematically as described below.

Starting from any primary package, count and number all the containers in numerical order as 1, 2, 3, 4.....up to r (defined below, and withdraw every r^{th} container thus counted to give a sample for the test, where:

r = integral part of N/n

N = the lot size

n = the number of primary packages to be selected (sample size)

7.2.2 Where primary packages are grouped into secondary packages, as far as it is possible, only one primary package from each secondary package shall be selected until the entire sample has been collected. Every effort shall be made to collect sample primary packages representative of all packing positions in the secondary packages. The selection of secondary packages for the sampling exercise shall be carried out in accordance with 7.3.

7.2.3 Where primary packages are sampled directly off the production line or not grouped into secondary packages, the lot shall consist of filled packages of detergent from the same batch and

date of manufacture. The sample size, n , shall be determined using Table 1. To obtain the laboratory samples, every i^{th} primary package of detergent shall be selected.

7.2.4 From each package of detergent sampled, the required amount of detergent (as specified in D.6) shall be removed first for microbiological examination as specified in clause 8. The remainder of the detergent shall undergo physical and chemical tests as specified in clause 8.

7.3 Selection of secondary packages

7.3.1 Where the number of secondary packages exceeds the sample size, n , the secondary packages will be selected randomly for inclusion in the sampling exercise.

7.3.2 Where the sample size, n , exceeds the number of secondary packages, every effort shall be made to apportion sampling quotas equally among the secondary packages of the lot.

8 Test methods

8.1 The rinsing property of the detergent shall be determined using the method specified in Annex A.

8.2 The type of surface active agent present shall be determined using the method specified in Annex B.

8.3 The total percentage of surface active agent present shall be determined by adding the results of clauses 8.3.1, 8.3.2, 8.3.3 and 8.3.4.

8.3.1 The percentage of anionic surfactant present shall be determined in accordance with ISO 2271:1989.

8.3.2 The percentage of cationic surfactant present shall be determined by adding the percentages obtained from the results of the test methods outlined in ISO 2871-1:1988 and ISO 2871-2:1988.

8.3.3 The percentage of non-ionic surfactant present shall be determined in accordance with the test method outlined in BS 3762:Section 3.7:1986.

8.3.4 The percentage of amphoteric surfactant present shall be determined in accordance with BS EN 13270:2001.¹

8.4 The level of coliforms shall be determined by the method specified in Annex D.

8.5 The level of yeasts and moulds shall be determined by the method specified in Annex E.

8.6 The presence of Gram-negative bacteria shall be determined by the method specified in Annex F.

¹ It is indicated within BS EN 15109:2006, *Surface active agents – Determination of the active matter content of alkylamidopropylbetaines* that BS EN 13270:2001 is statistically equivalent to BS EN 15109:2006 for the analysis of active matter content in alkylamidopropylbetaines. BS EN 13270 was selected because the reagents used were less hazardous than those used in BS EN 15109:2006.

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8.7 The biodegradability of the detergent shall be determined in accordance with ASTM D 2667-95 (2008).

9 Conformity assessment

9.1 The lot shall be considered to conform to the requirements of this national standard if the test sample, selected in accordance with clause 7.1, satisfies all the requirements of clauses 4, 5 and 6.

9.2 If a sample fails to meet one or more of the requirements of clauses 4, 5 and 6 of this national standard, the lot from which the samples were taken shall be considered as not complying.

Public Comment

Annex A (normative)

Determination of rinsing properties

A.1 Preparation of standard hard water

Dissolve 0.220 g chemically pure calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) and 0.246 g of chemically pure magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) in distilled water and dilute to 5 L with distilled water. (This standard hard water has a hardness of approximately 50 $\mu\text{g/g}$ calculated as calcium carbonate).

NOTE The standard hard water may alternatively be obtained from an external source.

A.2 Procedure

A.2.1 Accurately transfer (by pipette or syringe) 0.4 mL of the detergent into a thoroughly cleaned 500-mL flask. Add 200 mL of the standard hard water prepared as described in A.1, stopper the flask and shake vigorously for 1 minute.

A.2.2 Discard the solution and rinse the flask by adding 200 mL of the standard hard water and shaking vigorously for 1 minute. Repeat this rinsing once more. Invert the flask and allow to dry.

A.2.3 Carry out a blank determination by repeating the above procedure but omitting the sample.

A.2.4 Visually compare the two flasks.

A.3 Interpretation of results

The detergent is considered to be free-rinsing if the sediment, streaks and marks on the flask used for the test do not exceed those on the flask used in the blank determination.

Annex B (normative)

Qualitative test method for the determination of type of surface active agent present

B.1 Reagents

The reagents shall be of a recognized analytical grade. Water conforming to ASTM D1193-06, *Standard specification for reagent water* shall be used throughout.

B.1.1 Chloroform

B.1.2 Ammonium hexathiocyanatocobaltate solution

Dissolve 20 g of ammonium thiocyanate and 3 g of cobalt nitrate in about 70 mL of water and dilute to 100 mL.

B.1.3 Mixed Indicator

B.1.3.1 Dimidium bromide/acid blue 1 stock solution

This solution is prepared from acid blue 1 (CI 42045) (sodium α -[4-(diethylamino)phenyl]- α -[4-(diethylimino)cyclohexa-2,5-dienylidene]toluene-2,4-disulphonate) and dimidium bromide (3,8-diamino-5-methyl-6-phenyl-phenanthridinium bromide).

NOTE This solution is available commercially under the name 'dimidiumbromide-disulphine blue indicator stock solution'.

Weigh (0.5 ± 0.005) g of dimidium bromide into a 50-mL beaker and (0.25 ± 0.005) g of acid blue 1 into a second 50-mL beaker. Add between 20 mL and 30 mL of hot 10 % (v/v) ethanol to each beaker. Stir until dissolved and transfer the solution to a 250-mL one-mark volumetric flask with the ethanol solution and dilute to the mark with 10 % (v/v) ethanol.

B.1.3.2 Mixed acid indicator solution

Add 200 mL of water to 20 mL of the stock solution (B.1.3.1) in a 500-mL one-mark volumetric flask. Add 20 mL of the sulphuric acid solution, mix and dilute to the mark with water. Store out of direct sunlight.

B.1.4 Sodium hydroxide solution, $c(\text{NaOH}) = 5 \text{ mol/L}$

B.1.5 Sulphuric acid solution, $c(\text{H}_2\text{SO}_4) = 2.5 \text{ mol/L}$

B.2 Procedure

B.2.1 Preparation of test solution

Mix approximately 5 g of the detergent with 95 mL of water until all soluble components appear to have been dissolved. Filter to remove any insoluble matter.

NOTE It is not necessary that the filtrate be clear. If it is an emulsion, this condition will usually disappear and will not interfere with subsequent observations.

B.2.2 Test for type of surface active agent (acid medium)

Mix one drop of the test solution (B.2.1), 5 mL of the mixed acid indicator solution (B.1.3.2) and 5 mL of the chloroform (B.1.1). Allow the layers to separate. If the chloroform layer is not distinctly blue or pink, add a few more drops of the test solution, shake and allow separation. Repeat if necessary until about 1 mL of the test solution has been added.

A pink colour in the chloroform layer denotes the presence of a synthetic anionic-active surface active agent. A blue colour in the chloroform layer denotes the presence of a long chain amine, long chain amine oxide, or cationic active or ampholytic surface active agent. In the case of a blue colour or no colour, proceed in accordance with B.2.3.

B.2.3 Test for type of surface active agent (alkaline medium)

Mix one drop of the test solution (B.2.1), 5 mL of the mixed acid indicator solution (B.1.3.2), 1 mL of the sodium hydroxide solution (B.1.4) and 5 mL of the chloroform (B.1.1). Allow the layers to separate. If the chloroform layer is not distinctly blue or pink, add a few more drops of the test solution, shake and allow separation. Repeat if necessary until about 1 mL of the test solution has been added.

A pink colour in the chloroform layer denotes the presence of a soap or synthetic anionic-active or ampholytic surface active agent. Use the results from B.2.3 to distinguish these agents. A blue colour in the chloroform layer denotes the presence of a cationic active surface active agent.

B.2.4 Test for non-ionic surface-active agents

Mix 1 mL of the test solution (B.2.1), 10 mL of the ammonium hexathiocyanatocobaltate solution (B.1.2) and 5 mL of the chloroform (B.1.1). Allow the layers to separate.

A blue colour in the chloroform layer (in case of doubt, compare with a blank test, omitting the test solution) denotes the presence of a non-ionic surface active agent.

NOTE Non-ionic surface active agents that contain less than three ethylene oxide units per molecule, including those not based on ethylene oxide, will not be detected by this test.

Annex C (normative)

General guidance for the preparation of dilutions for microbiological examination

C.1 Principle

Preparation of the initial suspension is done in such a way as to obtain as uniform a distribution as possible of the micro-organisms contained in the test portion.

Preparation, if necessary, of decimal dilutions is done in order to reduce the number of micro-organisms per unit volume to allow, after incubation, observation of their growth (in the case of tubes or bottles) or colony counting (in the case of plates).

The appropriate number of micro-organisms is generally, for the colony count technique: 30 to 300 colonies (for some groups, for example coliforms, 15 to 150 colonies).

C.2 Diluent

C.2.1 Basic materials

In order to improve the reproducibility of the results, it is recommended that, for the preparation of the diluent, dehydrated basic components or a dehydrated complete preparation should be used. The manufacturer's instructions shall be rigorously followed.

Chemical products shall be of recognized analytical quality.

The water used shall be water distilled from glass apparatus, or deionised water. It shall be free from substances that might inhibit the growth of micro-organisms under the test conditions. This shall be periodically checked, particularly in the case of deionised water.

C.2.2 Composition

Unless there is irrefutable evidence (for example authoritative data or comparative tests) that other diluents are better suited for the preparation of particular products, use diluent with the following composition:

- a) peptone 1.0 g
- b) sodium chloride 8.5 g
- c) water 1 000 mL

C.2.3 Preparation

Dissolve the components in the water, by heating if necessary.

Adjust the pH so that, after sterilization, it is 7.0 pH units at 25 °C.

C.2.4 Distribution of diluent

Dispense the diluent (C.2.3) into test tubes or bottles (C.3.5) (for decimal dilutions) or into flasks or bottles (C.3.4) (for the initial suspension); (in the case of non-liquid products, see C.6.1.2) of appropriate capacities, in quantities such that, after sterilization, each tube or bottle contains 9.0 mL of diluent or a multiple of 9.0 mL and each flask or bottle contains 90 mL of diluent or a multiple of 90 mL (or other required quantities). Stopper the tubes, flasks, or bottles.

Sterilize by autoclaving at (121 ± 1) °C for 20 min.

If the diluent is not to be used immediately, store in the dark at a temperature between 0 °C and 5 °C for no longer than 1 month, in conditions which do not allow any change in its volume or composition.

NOTE If it is necessary to count several groups of micro-organisms using different culture media, it may be necessary to distribute all the dilutions (or some of them) in quantities greater than 9.0 mL. The size of the test tubes, flasks and bottles should be specified accordingly.

C.3 Apparatus and glassware

Usual microbiological laboratory equipment and in particular the equipment listed below.

NOTE Disposable apparatus is an acceptable alternative to reusable glassware, if it has suitable specifications. Glassware should be capable of undergoing repeated sterilization and should be chemically inert.

C.3.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave) (autoclave operating either separately or as part of an apparatus for preparing and distributing media).

Apparatus that will come into contact with the diluent, the sample, or the dilution, except for apparatus that is supplied sterile (plastic bags, plastic pipettes, etc.) shall be sterilized by one of the following methods:

- a) by being kept at 170 °C to 175 °C for not less than 1 h in an oven;
- b) by being kept at (121 ± 1) °C for not less than 20 min in an autoclave.

C.3.2 Blending equipment (for non-liquid products, see C.6.1.2).

One of the following shall be used:

- a rotary blender, operating at between 8 000 and 45 000 revolutions per minute, with glass or metal bowls preferably fitted with lids, resistant to the conditions of sterilization;
- a peristaltic-type blender (stomacher), with sterile plastic bags.

NOTE The bowls or plastic bags should have sufficient capacity to allow the sample to be properly mixed with the appropriate amount of diluent. In general, the volume of the container should be equal to about twice the volume of the sample plus diluent.

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C.3.3 Mixer

Mixer capable of mixing 1 or 2 mL of the sample (in the case of liquid products), or of a higher dilution, in a tube of adequate dimensions, with 9 or 18 mL of diluent, in order to obtain a homogeneous suspension, and working on the principle of eccentric rotation of the contents of the test tube (Vortex mixer).

C.3.4 Flasks

Flasks of sufficient capacity to contain the 90 mL of diluent used for the initial suspension, or multiples of 90 mL (in the case of non-liquid products, see C.6.1.2).

C.3.5 Test tubes

Test Tubes of sufficient capacity to contain, and leave adequate head-space for mixing, 10 mL (or a multiple of 10 mL, if necessary) of the sample (if liquid) or of the initial suspension (in other cases), or further decimal dilutions.

C.3.6 Pipettes

Pipettes (plugged with cotton wool), of nominal capacity 1 mL (or if necessary 2 mL; see the note to C.2.4) and having an outlet of diameter 2 to 3 mm.

C.3.7 Graduated pipettes

Graduated pipettes (plugged with cotton wool), of large capacity, for example 10 to 20 mL

C.3.8 pH meter

pH meters accurate to ± 0.1 pH unit

C.3.9 Balance

Balance of sufficient capacity, capable of weighing to the nearest 0.01 g (in the case of non-liquid products)

C.4 Sampling

Sampling shall be conducted in such a way as to obtain a representative test sample of the product. See clause 7.

C.5 Preparation of the test sample

Thoroughly mix the laboratory samples for liquid, gel and paste detergents before taking representative test samples. Use the test samples immediately upon removal from the package.

Sample the bar detergent using aseptic techniques to bore through the bar to obtain a core of the product.

C.6 Procedure

C.6.1 Test portion and initial suspension (primary dilution)

To avoid damaging the micro-organisms by sudden changes in temperature, the temperature of the diluent during the operations described below shall be approximately the same as that of the test sample.

C.6.1.1 Liquid samples (which can be taken by pipette)

Shake the test sample manually by performing 25 up and down movements of amplitude about 30 cm in 7 s, or preferably use a standardized mechanical device to ensure uniform distribution of micro-organisms. Take 1 mL with a pipette (C.3.6) and add this test portion to 9 mL of diluent (C.2.4) avoiding contact between the pipette and the diluent (see the note to C.2.4).

Carefully mix the test portion and diluent, either by aspirating ten times with a different pipette, or in the mechanical mixer (C.3.3) for 5 to 10 s. The frequency of rotation of the latter shall be chosen so that the liquid, as it swirls, rises to within 2 or 3 cm of the rim of the vessel.

Carefully mix the test portion and diluent, either aspirating ten times with a different pipette, or in the mechanical mixer (C.3.3) for 5 to 10 s. the frequency of rotation of the latter shall be chosen so that the liquid, as it swirls, rises to within 2 or 3 cm of the rim of the vessel.

C.6.1.2 Other samples

Weigh, to the nearest 0.01 g into a bowl (in the case of the rotary blender (C.3.2a)) or in a plastic bag (in the case of the stomacher (C.3.2b)), a mass (m) (generally 10 g or a multiple of 10 g) of the test sample (see clause C.5), of sufficient size so that all the tests and all the further dilutions appropriate to the product concerned can be performed.

Add a volume, in millilitres, equal numerically to $9 \times m$ of the diluent (C.2.4) at the appropriate temperature.

NOTE It may be necessary, in certain cases, particularly for products giving an initial 1 + 9 suspension, which is too viscous or too thick, to add more diluent. This should be taken into account for subsequent operations and/or in the expression of results.

Operate the rotary blender for a sufficient time to give a total number of 15 000 to 20 000 revolutions. Even with the slowest blender, this time shall not exceed 2.5 min.

Operate the stomacher for 1 to 2 min.

Allow larger particles to settle, if necessary for up to 15 min, then transfer a certain quantity from the top layer of the suspension to a culture tube, flask, or bottle (C.3.5) using a large pipette (C.3.7) (if there is a fat layer, take the sample from the aqueous part).

This quantity shall be of sufficient size so that all the tests and further dilutions can be performed. If only one portion has to be taken from the initial suspension for inoculation or further dilution, this transfer may be omitted.

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C.6.2 Further decimal dilutions

In the case of a presence or absence test for a micro-organism in 0.1 mL or 0.1 g of product, it is not necessary to prepare the following dilutions.

Transfer, by means of a fresh pipette (or, if the mixture of the initial suspension was obtained using a pipette, use the same pipette), 1 mL of the initial suspension (C.6.1.1 or C.6.1.2) into another tube containing 9 mL of sterile diluent at the appropriate temperature, avoiding contact between the pipette and the diluent (see the notes to C.2.4).

Mix carefully, either by aspirating ten times with a fresh pipette or in the mechanical mixer (C.3.3) for 5 to 10 s, to obtain a 10^{-2} dilution. The frequency of rotation of the latter shall be chosen so that the liquid, as it swirls, rises to within 2 or 3 cm of the rim of the vessel.

If necessary, repeat these operations using the 10^{-2} and further dilutions to obtain 10^{-3} , 10^{-4} , etc., number of micro-organisms has been obtained (see clause C.1).

C.6.3 Repetition of the different operations

Carry out the series of operations described in C.6.1 and C.6.2 as many times as is required (once, twice, etc.).

NOTE It has been established statistically that, in order to reduce the variability of the results when using the colony count technique, it is preferable to repeat the different operations with separate portions of test sample rather than to double the number of plates inoculated from each tube of a single dilution series.

C.6.4 Duration of the procedure

In general, the dilutions shall be prepared from the test sample immediately prior to the analysis; they shall be used for inoculating culture media within 30 min of preparation.

Annex D (normative)

General guidance for the enumeration of coliforms – colony count technique

D.1 Principle

Preparation of two poured plates, using a solid selective culture medium, and using a specified quantity of the test sample if the initial product is liquid, or using a specified quantity of an initial suspension in the case of other products

Preparation of other pairs of poured plates, under the same conditions, using decimal dilutions of the test sample or of the initial suspension

Incubation of the plates at 37 °C for 24 h

Calculation of the number of coliforms per millilitre or per gram of sample from the number of characteristic colonies obtained in the plates chosen (see D.7.1)

D.2 Culture medium and dilution fluid

D.2.1 Dilution fluid

See Annex C.

D.2.2 Solid selective medium: crystal violet neutral red bile lactose (VRBL) agar

Composition

a) peptone	7 g
b) yeast extract	3 g
c) lactose	10 g
d) sodium chloride	5 g
e) bile salts	1.5 g
f) neutral red	0.03 g
g) crystal violet	0.002 g
h) agar	12 g to 18 g
i) water	1 000 mL

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D.2.3 Preparation

Proceed as follows in order to conserve the selective power and specificity of the medium.

Thoroughly mix the components of the dehydrated complete medium in the water and leave to stand for several minutes. Adjust the pH so that, after boiling, it is 7.4 at 25 °C. Bring to the boil, stirring from time to time.

Allow to boil for 2 min. Immediately cool the medium in the water-bath (D.3.5) set at 45 °C.

Avoid overheating the medium, heating for too long or reheating it. Consequently, do not sterilize in the autoclave, and check the sterility of the medium at the time of use (see D.6.2.2).

D.3 Apparatus and glassware

Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment and, in particular, the equipment listed below.

D.3.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave)

Autoclave capable of reaching temperatures of greater than 121 °C equipped with one or more safety valves, pressure gauge, draincock, temperature regulating device (± 1 °C), thermometer or recording thermocouple.

An oven capable of maintaining temperatures of 170 °C to 180 °C for 1 h equipped with a thermostat, thermometer or recording thermocouple.

D.3.2 Incubator, capable of operating at (37 ± 1) °C

D.3.3 Petri dishes made of glass or plastic, of diameter 90 mm to 100 mm

D.3.4 Total delivery pipettes, having a nominal capacity of 1 mL

D.3.5 Water-bath, or similar apparatus, capable of operating at (45 ± 0.5) °C

D.3.6 Colony counting equipment, consisting of an illuminated base and a mechanical or electronic digital counter

D.3.7 pH meter, accurate to ± 0.1 pH unit at 25 °C

D.4 Sampling

Sampling shall be conducted in such a way as to obtain a representative test sample of the product. See clause 7.

D.5 Preparation of the test sample

Thoroughly mix the laboratory samples for liquid, gel and paste detergents before taking representative test samples. Use the test samples immediately upon removal from the package.

Sample the bar detergent using aseptic techniques to bore through the bar to obtain a core of the product.

D.6 Procedure

D.6.1 Test portion, initial suspension and dilutions

See Annex C.

D.6.2 Inoculation and incubation

D.6.2.1 Take two sterile Petri dishes (D.3.3). Using a sterile pipette (D.3.4), transfer to each dish 1 mL of the test sample, if the product is liquid, or 1 mL of the initial suspension in the case of other products.

Take two other sterile Petri dishes. Using a fresh sterile pipette, transfer, to each dish, 1 mL of the first decimal dilution (10^{-1}) of the test sample (if the product is liquid) or 1 mL of the first decimal dilution (10^{-2}) of the initial suspension (in the case of other products).

Repeat the procedure described with the further dilutions, using a fresh sterile pipette for each decimal dilution.

D.6.2.2 Pour about 15 mL of the VRBL medium (D.2.2), at $(45 \pm 0.5) ^\circ\text{C}$, into each Petri dish. The time elapsing between the end of the preparation of the initial suspension (or of the 10^{-1} dilution of the product is liquid) and the moment when the medium (D.2.2) is poured into the dishes shall not exceed 15 min.

Carefully mix the inoculum with the medium and allow the mixture to solidify, with the Petri dishes standing on a cool horizontal surface.

Also prepare a control plate, with 15 mL of the medium for checking its sterility.

D.6.2.3 After complete solidification, pour about 4 mL of the VRBL medium (D.2.2), at $(45 \pm 0.5) ^\circ\text{C}$, on to the surface of the inoculated medium. Allow to solidify as described above.

D.6.2.4 Invert the prepared dishes and incubate them in the incubator set at $37 ^\circ\text{C}$ for (24 ± 2) h.

D.6.3 Counting of the colonies

After the specified period of incubation (see D.6.2.4), count, using the colony counting equipment (D.3.6), the characteristic coliform colonies in each dish containing not more than 150 colonies whether characteristic or not.

NOTE After incubation for 24 h, characteristic colonies are purplish red colonies having a diameter of 0.5 mm or greater and sometimes surrounded by a reddish zone of precipitated bile.

D.7 Expression of results

D.7.1 Method of calculation

D.7.1.1 Dishes containing between 15 and 150 characteristic colonies

Retain dishes containing not more than 150 characteristic colonies at two consecutive dilutions. It is necessary that one of these dishes contains at least 15 characteristic colonies.

Calculate the number N of coliforms per millilitre or per gram of product, depending on the case, using the following equation:

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

where $\sum C$ is the sum of the characteristic colonies counted on all the dishes retained;

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

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

d is the dilution factor corresponding to the first dilution.

Round the result calculated to two significant figures.

Take as the result the number of coliforms per millilitre or per gram of product, expressed as a number between 1.0 and 9.9 multiplied by 10^x , where x is the appropriate power of 10.

EXAMPLE 1 A coliform count at 30 °C gave the following results:

- at the first dilution retained (10^{-2}): 83 and 97 characteristic colonies
- at the second dilution retained (10^{-3}): 13 and 8 characteristic colonies

$$N = \frac{\sum C}{(n_1 + 0.1n_2)d} = \frac{83 + 97 + 13 + 8}{(2 + 0.1 \times 2) \times 10^{-2}} = \frac{201}{0.022} = 9136$$

Rounding the result as specified above gives 9 100 or 9.1×10^3 coliforms per millilitre or per gram of product.

D.7.1.2 Dishes containing less than 15 characteristic colonies

If each of the dishes retained contains less than 15 characteristic colonies, calculate the estimated number (N_E) of coliforms using the equation given in D.7.1.1.

EXAMPLE 2 A coliform count at 30 °C gave the following results:

- at the 10^{-4} dilution: 140 and 145 colonies, of which 5 and 3 colonies respectively were characteristic
- at the 10^{-5} dilution: 11 and 8 colonies, of which 0 and 1 colonies respectively were characteristic

$$N_E = \frac{(5 + 3 + 0 + 1)}{(2 + 0.1 \times 2) \times 10^{-4}} = \frac{9}{2.2 \times 10^{-4}} = 40000$$

Rounding the result as specified in D.7.1.1 gives 4.0×10^4 coliforms per millilitre of per gram of product.

D.7.1.3 Estimation of small numbers

If the two dishes, corresponding to the test sample (liquid products) or the initial suspension (other products), contain less than 15 characteristic colonies, report the result as follows:

- less than 15 coliforms per millilitre (liquid products);
- less than $15 \times 1/d$ coliforms per gram (other products), where d is the dilution factor of the initial suspension.

D.7.1.4 Dishes containing no characteristic colonies

If the two dishes, corresponding to the test sample (liquid products) or the initial suspension (other products), contain no characteristic colonies, report the result as follows:

- less than 1 coliform per mL (liquid products);
- less than $1 \times 1/d$ coliforms per gram (other products), where d is the dilution factor of the initial suspension.

D.7.2 Precision

D.7.2.1 Dishes containing between 15 and 150 characteristic colonies (see D.7.1.1)

For statistical reasons alone, in 95 % of cases the confidence limits of this method vary from ± 16 % to ± 52 %.² In practice, even greater variation may be found especially among results obtained by different microbiologists.

D.7.2.2 Each dish contains less than 15 characteristic colonies (see D.7.1.2)

Refer to Table 1. To obtain the confidence limits, multiply the lower and upper limits given by $1/d$, where d is the dilution factor.

D.7.2.3 Estimation of small numbers (see D.7.1.3)

The confidence limits for the estimation of small numbers of coliforms are given in table D.1.

D.7.2.4 Confidence limits for the estimation of small numbers of colonies

The confidence limits at the 95 % level for the estimation of small numbers, when the number of characteristic colonies on dishes retained is less than 15, are given in Table D.1.

² (Cowell and Morisetti, *J. Sic. Fd. Agric.*, 1969, Vol. 20, p. 573)

Table D.1 – Confidence limits

Number of coliforms	Confidence limits at the 95 % level	
	lower	upper
1	<1	2
2	<1	4
3	<1	5
4	1	6
5	2	9
6	2	10
7	2	12
8	3	13
9	4	14
10	4	16
11	5	18
12	6	19
12	7	20
14	7	21
15	8	23

Public Comment

Annex E (normative)

General guidance for enumeration of yeasts and moulds – colony count techniques at 25 °C

E.1 Principle

Preparation of poured plates using a specified selective culture medium and a specified quantity of the test sample if the initial product is liquid, or of an initial suspension in the case of other products

Preparation of other plates, under the same conditions, using decimal dilutions of the test sample or of the initial suspension

Aerobic incubation of the plates at 25 °C for 3, 4 or 5 days

Calculation of the number of yeasts and moulds per gram or per millilitre of sample from the number of colonies obtained on plates chosen at dilution levels so as to give a significant result

E.2 Diluent and culture medium

E.2.1 Basic materials

In order to improve the reproducibility of the results, it is recommended that, for the preparation of the culture medium, dehydrated basic components or a complete dehydrated medium be used. The manufacturer's instruction shall be rigorously followed.

The chemical products used shall be of recognized analytical quality.

The water used shall be distilled or deionised water, free from substances that might inhibit the growth of yeasts and moulds under the test conditions.

Measurements of pH shall be made using a temperature-compensated pH meter (E.3.4).

If the prepared diluent and culture medium are not used immediately, they shall, unless otherwise stated, be stored in the dark at between 0 °C and 5 °C, for no longer than 1 month, in conditions which do not produce any change in their composition.

E.2.2 Diluent

See Annex C.

E.2.3 Yeast extract-dextrose-chloramphenicol-agar medium

Table E.1 – Composition of medium

Component	Quantity
Yeast extract	5g
Dextrose (C ₆ H ₁₂ O ₆)	20 g
Chloramphenicol (C ₁₁ H ₁₂ Cl ₂ N ₂ O ₅)	0.1 g ^a
Agar	12 g to 15 g ^b
Water	1000 mL

^a In order to obtain a final concentration of 100 µg/mL of medium.
^b According to the manufacturer's instructions.

Dissolve the components in the water by boiling.

If necessary adjust the pH so that after sterilization it is 6.6.

Dispense the agar medium into suitable containers (See E.3.5).

Sterilize at (121 ± 1) °C for 15 min.

E.3 Apparatus and glassware

NOTE Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment, and in particular the equipment listed below

E.3.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave)

The autoclave may either operate separately or be part of a general apparatus for the preparation and distribution of media.

Sterilize apparatus that will come into contact with the diluent, the culture medium or the sample, particularly plastics apparatus, except for apparatus that is supplied sterile, by one of the following methods:

- in the oven by maintaining it at (170 to 175) °C for not less than 1 h; or
- in the autoclave by maintaining it at (121 ± 1) °C for not less than 20 min.

E.3.2 Incubator, capable of being maintained at (25 ± 1) °C.

E.3.3 Water-bath, capable of being maintained at (45 ± 1) °C.

E.3.4 Temperature-compensated pH meter, having an accuracy of calibration of ± 0.1 pH unit at 25 °C.

E.3.5 Culture bottles or flasks

Bottles or flasks with non-toxic metal screw-caps may be used.

E.3.6 Graduated pipettes, calibrated for bacteriological use only, of nominal capacities 10 and 1 mL, graduated in divisions of 0.5 and 0.1 mL respectively, and with an outflow opening of 2 mm to 3 mm.

E.3.7 Petri dishes, of diameter 90 mm to 100 mm.

E.4 Sampling

Sampling shall be conducted in such a way as to obtain a representative test sample of the product. See clause 7.

E.5 Preparation of the test sample

Thoroughly mix the laboratory samples for liquid, gel and paste detergents before taking representative test samples. Use the test samples immediately upon removal from the package.

Sample the bar detergent using aseptic techniques to bore through the bar to obtain a core of the product.

E.6 Procedure

E.6.1 Test portion, initial suspension and dilutions

See Annex C.

E.6.2 Inoculation and incubation

E.6.2.1 Take two sterile Petri dishes (E.3.7). Transfer to each dish, by means of a sterile pipette (E.3.6), 1 mL of the test sample if liquid, or 1 mL of the initial suspension in the case of other products.

E.6.2.2 Take two further Petri dishes. Transfer, by means of another sterile pipette, 1 mL of the 10^{-1} dilution to each dish (liquid product), or 1 mL of the 10^{-2} dilution (other product).

Repeat the procedure described above using further dilutions if necessary.

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E.6.2.3 Pour about 15 mL of the yeast extract-dextrose-chloramphenicol-agar medium (E.2.3), previously melted and maintained at $45^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in a water-bath (E.3.3), from a culture bottle (E.3.5) into each Petri dish. The time elapsing between the end of the preparation of the initial suspension (or of the 10^{-1} dilution if the product is liquid) and the moment when the medium is poured into the dishes shall not exceed 15 min.

Carefully mix the inoculum with the medium and allow the mixture to solidify, by leaving the Petri dishes to stand on a cool horizontal surface.

Prepare a control plate, with 15 mL of the medium, to check its sterility.

E.6.2.4 Invert the plates and place them in the incubator (E.3.2) at $(25 \pm 1)^{\circ}\text{C}$.

E.6.3 Interpretation

Count the colonies on each plate after 3, 4 and 5 days of incubation. After 5 days, retain those plates containing fewer than 150 colonies. If parts of the plates are overgrown with moulds, or if it is difficult to count well-isolated colonies, retain the counts obtained after 4 or even 3 days of incubation. In this event, record the incubation period of 3 or 4 days in the test report.

If necessary, carry out a microscopic examination in order to distinguish, according to their morphology, the colonies of yeasts and moulds from colonies of bacteria.

E.7 Expression of results

E.7.1 Calculation

E.7.1.1 Use counts from plates containing fewer than 150 colonies.

E.7.1.2 The number of yeasts and moulds per gram or per millilitre is equal to

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

where $\sum C$ is the sum of colonies counted on all the plates;

n_1 is the number of plates counted in the first dilution;

n_2 is the number of plates counted in the second dilution;

d is the dilution from which the first counts were obtained (e.g. 10^{-2}).

E.7.1.3 Round the result obtained in E.7.1.2 to two significant figures. When the number to be rounded is 5, with no further significant figures, round the number to give an even figure immediately to the left, for example 28 500 is rounded to 28 000; 11 500 is rounded to 12 000.

E.7.1.4 The result shall be expressed as a number between 1.0 and 9.9 multiplied by 10^x , where x is the appropriate power of 10.

If there were no colonies on plates from the initial suspension (E.6.1), if the initial product was solid, the number of yeasts and moulds per gram of product should be reported as fewer than 10.

If there were no colonies on plates from the test sample, if the initial product was liquid (E.6.1), the number of yeasts and moulds per millilitre of product should be reported as fewer than 1.

EXAMPLE A yeasts and moulds count gave the following results (two Petri dishes per dilution were incubated):

10^{-2} dilution: 83 and 97 colonies

10^{-3} dilution: 33 and 28 colonies

$$\frac{\sum C}{(n_1 + 0.1n_2)d} = \frac{(83 + 97 + 33 + 28)}{[2 + (0.1 \times 2)] \times 10^{-2}} = \frac{241}{0.022} = 10954$$

Rounding the result as specified in E.7.1.3 gives 11 000

The estimated number of yeasts and moulds per gram or per millilitre is therefore 1.1×10^4 .

E.7.2 Precision

For statistical reasons in 95 % of cases the confidence levels for this method vary from ± 16 % to ± 52 %.

NOTE In practice, even larger variations may be observed, in particular between the results obtained by different microbiologists.

Annex F
(normative)

Qualitative test method for the determination of the presence of Gram-negative bacteria

F.1 Reagents

F.1.1 Hucker's crystal violet

F.1.1.1 Solution A

- | | |
|-------------------------------------|-------|
| a) Crystal violet (90% dye content) | 2 g |
| b) Ethanol, 95% | 20 mL |

F.1.1.2 Solution B

- | | |
|---------------------|-------|
| a) Ammonium oxalate | 0.8 g |
| b) Distilled water | 80 mL |

F.1.1.3 Mix solution A and B. Store for 24 h and filter through coarse filter paper

F.1.2 Gram's iodine

- | | |
|--------------------------|--------|
| a) Iodine | 1 g |
| b) Potassium iodine (KI) | 2 g |
| c) Distilled water | 300 mL |

Place KI in mortar, add iodine, and grind with pestle for 5 s to 10 s. Add 1 mL water and grind; then add 5 mL of water and grind, then 10 mL of water and grind. Pour this solution into a reagent bottle. Rinse mortar and pestle with amount of water needed to bring total volume to 300 mL.

F.1.3 Hucker's counterstain (stock solution)

- | | |
|---------------------------|--------|
| a) Safranin O (certified) | 2.5 g |
| b) Ethanol, 95% | 100 mL |

Add 10 mL of the stock solution to 90 mL of distilled water and mix.

F.2 Staining procedure (Gram stain)

Fix air-dried films of sample in moderate heat. Stain films 1 min with crystal violet-ammonium oxalate solution. Wash briefly in tap water and drain. Apply Gram's iodine for 1 minute. Wash in tap water

and drain. Decolourize with 95% ethanol until washings are no longer blue (about 30 s). Alternately, flood slides with ethanol, pour off immediately, and reflood with ethanol for 10s. Wash briefly with water, drain, blot or air-dry and examine microscopically. Gram-positive bacteria stain blue; Gram-negative bacteria stain red.

Some Gram-negative bacteria do not destain readily after staining with Hucker's crystal violet. To avoid this difficulty, dilute crystal violet solution up to ten fold before mixing with equal parts of ammonium oxalate solution. Stain reference Gram-positive and Gram-negative bacteria to ensure valid staining results.

F.3 Requirements

The detergent is considered to contain no Gram-negative bacteria if no red stain is observed.

Public Comment