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PUBLICLY AVAILABLE SPECIFICATION

PRE-STANDARD

Clothes washing machines for household use – Method for measuring the microbial contamination reduction

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IEC Central Office
3, rue de Varembé
CH-1211 Geneva 20
Switzerland

Tel.: +41 22 919 02 11
Fax: +41 22 919 03 00
info@iec.ch
www.iec.ch

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CLOTHES WASHING MACHINES FOR HOUSEHOLD USE – METHOD FOR MEASURING THE MICROBIAL CONTAMINATION REDUCTION

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A bilingual version of this publication may be issued at a later date.

INTRODUCTION

SC 59D decided to address the measurement of the microbial contamination reduction in washing machines by developing a globally acceptable Publicly Available Specification to respond to the increase in consumer complaints regarding odour from washing machines and washed laundry caused by presence of microorganisms.

CLOTHES WASHING MACHINES FOR HOUSEHOLD USE – METHOD FOR MEASURING THE MICROBIAL CONTAMINATION REDUCTION

1 Scope

This Publicly Available Specification (PAS) specifies a test method for measuring the reduction of microbial contamination in clothes washing machines and of the possible cross contamination to uncontaminated load.

NOTE A significant differentiation in microorganism contamination reduction capability of washing machines can be expected at wash temperature not higher than 40 °C.

This PAS applies to clothes washing machines for household use, with or without heating devices utilising cold and/or hot water supply. It also covers washing machines which specify the use of no detergent for normal use. This PAS applies also to washing machines for communal use in blocks of flats or in launderettes.

This PAS does not deal with professional washing machines nor with commercial laundry operations associated with food service, hospital linens or other non-residential applications. It also does not address the needs of persons with specific health conditions requiring special sanitization and/or disinfection techniques.

This PAS does not specify safety requirements and does not deal with performance of washing machines measured under IEC 60456 nor with effects on fabrics.

2 Normative references

IEC 60456:2010, *Clothes washing machines for household use – Methods for measuring the performance*

ISO 2267, *Surface active agents – Evaluation of certain effects of laundering – Methods of preparation and use of unsoiled cotton control cloth*

EN 1276, *Chemical disinfectants and antiseptics. Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas. Test method and requirements (phase 2, step 1)*

EN 1650, *Chemical disinfectants and antiseptics. Quantitative suspension test for the evaluation of fungicidal or yeasticidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas. Test method and requirements (phase 2, step 1)*

EN 12353, *Chemical disinfectants and antiseptics. Preservation of test organisms used for the determination of bactericidal (including Legionella), mycobactericidal, sporicidal, fungicidal and virucidal (including bacteriophages) activity*

3 Terms, definitions and symbols

3.1 Terms and definitions

3.1.1
washing machine
appliance for cleaning and rinsing of textiles using water which may also have a means of extracting excess water from the textiles

3.1.2
test washing machine
washing machine that is subjected to part or all of the requirements in this PAS in order to determine its performance

3.1.3
test run
single performance assessment

3.1.4
programme
series of **operations** which are pre-defined within the **washing machine** and which are declared by the manufacturer as suitable for washing certain textile types

3.1.5
base load
textile load used for testing not including the **bio monitors**

3.1.6
cross contamination
transfer of microorganisms from fabrics to fabrics during one **test run**

3.1.7
inoculum
quantity of cells to be inoculated in culture medium

3.1.8
bio monitor
microorganism carrier inoculated with microorganisms to be used to monitor the reduction of microbial contamination

3.1.9
temperature profile
time–temperature data representing the water temperature in the **washing machine** during the **test run**

3.2 Symbols

N_0	average value of microorganism amount of the two positive controls, before exposition to the test **programme** (cfu/bio monitor)
N	average value of microorganism amount per 5 **bio monitors**, after exposition to the test **programme** (cfu/bio monitor)
$\log(N_0/N)$	reduction factor per microorganism type
v^{-1}	microorganism amount in a 10 times diluted solution
v^{-2}	microorganism amount in a 100 times diluted solution
$SD_{initial}$	standard deviation of N_0
SD_{washed}	standard deviation of N

SD_{total} standard deviation of the reduction factor per microorganism type

4 Requirements

This Publicly Available Specification specifies a test method for measuring the reduction of microbial contamination in clothes **washing machines** and of the possible cross contamination in subsequent washed loads.

This PAS does not specify safety requirements and does not deal with performance of **washing machines** measured under IEC 60456 nor with effects on fabrics.

5 Test conditions, materials, equipment and instrumentation

5.1 Test conditions

For all requirements regarding ambient conditions (electricity supply, water supply, ambient temperature and humidity) and **base load**, see IEC 60456:2010.

Additionally, the water supplied to the **test washing machine** shall not contain more than 100 cfu/ml. Microorganisms for test purposes as listed in 5.2.1 shall not be present in the water.

The micribiological quality of the water supplied to the **test washing machine** is determined according to 5.2.3.2 and 6.5.3

5.2 Materials and reagents

5.2.1 Microorganisms for test purposes

For test purposes the following microorganisms, the first two bacteria strains and the last one a yeast strain, shall be used:

- *Pseudomonas putida* (ATCC 11172 / DSM 6521)
- *Staphylococcus aureus* (ATCC 6538 / DSM 799)
- *Candida albicans* (ATCC 10231 / DSM 1386)

NOTE Additional microorganisms may be used. For internal development purpose and pre-evaluation of microorganism contamination reduction in clothes **washing machines**, a protocol with risk class 1 test microorganisms may be performed as described in Annex A.

5.2.2 Culture media and solutions

5.2.2.1 Culture media

All media and solutions shall be of microbiology grade and sterilized appropriately prior to use. It is recommended to use commercially available and/or water-free dry materials for the culture media. The specifications in the following clauses refer to water-free products.

5.2.2.1.1 Tryptone Soy Agar (TSA)

The composition of Tryptone Soy Agar (TSA) shall be according to Table 1.

Table 1 – Composition of Tryptone Soy Agar (TSA)

Description	Specification
Casein peptone (pancreatic digest)	15,0 g/l
Soy peptone (papaic digest)	5,0 g/l
Sodium chloride	5,0 g/l
Agar	15,0 g/l
Final pH	7,3 ± 0,2

5.2.2.1.2 Sabouraud Dextrose Agar with Chloramphenicol

The composition of Sabouraud Dextrose Agar with Chloramphenicol shall be according to Table 2.

Table 2 – Composition of Sabouraud Dextrose Agar with Chloramphenicol

Description	Specification
Pancreatic digest of casein	5,0 g/l
Peptic digest of animal tissue	5,0 g/l
Dextrose monohydrate	40,0 g/l
Chloramphenicol	0,05 g/l
Agar	15,0 g/l
Final pH	5,6 ± 0,2

5.2.2.1.3 Cetrimide Agar

The composition of Cetrimide Agar shall be according to Table 3.

Table 3 – Composition of Cetrimide Agar

Description	Specification
Pancreatic digest of gelatine	20,0 g/l
Magnesium chloride	1,4 g/l
Potassium sulphate	10,0 g/l
Glycerine	10,0 ml/l
Cetrimide	0,2 g/l
Agar	14,0 g/l
Final pH	7,1 ± 0,2

5.2.2.1.4 Baird-Parker Agar

The composition of Baird-Parker Agar shall be according to Table 4.

Table 4 – Composition of Baird-Parker Agar

Description	Specification
Casein peptone (pancreatic digest)	10,0 g/l
Beef extract	5,0 g/l
Yeast extract	1,0 g/l
Lithium chloride	5,0 g/l
Glycine	12,0 g/l
Sodium pyruvate	10,0 g/l
Agar	20,0 g/l
Sodium tellurite	0,1 g/l
Egg yolk emulsion	50,0 ml/l
Final pH	6,8 ± 0,2

5.2.2.1.5 Malt Extract Agar (MEA)

The composition of Malt Extract Agar (MEA) shall be according to Table 5.

Table 5 – Composition of Malt Extract Agar (MEA)

Description	Specification
Malt extract	30,0 g/l
Soy-peptone, which is digested with papain from soybeans	3,0 g/l
Agar	15,0 g/l
Final pH	5,6 ± 0,2

5.2.2.1.6 Tryptone Soy Broth (TSB)

The composition of Tryptone Soy Broth (TSB) shall be according to Table 6.

Table 6 – Composition of Tryptone Soy Broth (TSB)

Description	Specification
Tryptone, pancreatic digest of casein	17,0 g/l
Soy peptone, papaic digest of soybean meal	3,0 g/l
Sodium chloride (NaCl)	5,0 g/l
Dipotassium hygrogen phosphate (K_2HPO_4)	2,5 g/l
Glucose	2,5 g/l
Final pH	7,3 ± 0,2

5.2.2.2 Water for culture media and solutions

Bi-distilled or demineralised water can be used. However, demineralised water shall be sterilised prior to use.

5.2.2.3 Diluting agent

The composition of the diluting agent shall be as in Table 7.

Table 7 – Composition of diluting agent

Description	Specification
Tryptone, pancreatic digest of casein	1,0 g/l
Sodium chloride (NaCl)	8,5 g/l
Polysorbate 80	(0,05 %)
Final pH	7,0 ± 0,2

5.2.2.4 Neutralisation solution

For microorganisms growth neutralisation solution shall be used in combination with appropriate nutrition media.

The composition of a recommended neutralisation solution is given in Table 8.

Table 8 – Composition of recommended neutralisation solution

Description	Specification
Polysorbate 80	30,0 g/l
Lecithine	3,0 g/l
L-histidine	1,0 g/l
Natriumthiosulphate	5,0 g/l

Other neutralisation solutions may be used if it can be shown that they give the same results.

5.2.3 Detergent

5.2.3.1 General

The detergent shall be IEC A* base powder according to IEC 60456.

5.2.3.2 Pre test for water quantity

A pre test to determine the water quantity, which is the calculation base for the detergent dosage, is necessary. The following conditions shall apply:

a) Run 3 times the **programme** which is foreseen to be tested.

b) Use the same load size as in the test with **bio monitors**.

c) Use in all 3 tests dry **base load** (according to IEC 60456).

d) The detergent dose is 2 g per kg of **base load**.

e) The detergent shall be placed into the **washing machine** detergent dispenser. If there is no detergent dispenser, follow the manufacturer's instructions for detergent placement. If no instructions are given, all detergent is added into the **washing machine** at the base of the drum.

   The detergent is necessary to reduce the surface tension which can influence the soaking behaviour

f) Calculate the average water quantity for the main wash for the 3 repetitions of the **programme.** Water quantity in the main wash shall be determined according to IEC 60456.

5.2.3.3 Detergent dosage in main test

The detergent dosage shall be 2 g per litre of water in the main wash.

If a detergent dispenser is present, the detergent dose specified shall be placed in the dispenser.

If there is no detergent dispenser, follow the manufacturer's instructions for detergent placement. If no instructions are given, all detergent is added into the **washing machine** at the base of the drum.

5.3 Equipment

5.3.1 General

Test conditions, materials, equipment and instrumentation shall be in accordance with IEC 60456, unless otherwise specified, and handled in compliance with good microbiology laboratory practice.

5.3.2 Incubator

The incubator shall be capable of maintaining a constant temperature of (36 ± 1) °C for bacterial strains and (30 ± 1) °C for the yeast strain.

5.3.3 Autoclave

The autoclave shall be capable of sterilizing equipment and supplies by subjecting them to saturated steam at (121 +3/-0) °C for at least 15 min or at (134 +3/-0) °C for at least 5 min.

5.3.4 Microorganism carrier

The microorganism carriers are swatches made of standard cotton fabric in accordance with ISO 2267. The dimension shall be 1,0 cm × 1,0 cm.

The swatches shall be boiled 3 times in distilled water, autoclaved, dried at room temperature and kept sterile prior to use.

5.3.5 Pipettes

Pipettes shall have a nominal volume of 0,1 ml to 10,0 ml.

5.3.6 Electromechanical agitator

An electromechanical agitator commonly used in laboratories can be used for the purposes of this PAS. An example of a suitable commercially available product is given in Annex B.

5.3.7 Centrifuge, centrifuge tubes

The centrifuge shall be capable of maintaining and be used at a relative centrifugal force of 2 000 × g with 50 ml sterile centrifuge tubes.

5.3.8 Base load

The **base load** shall consist of cotton **base load** items as defined in IEC 60456:2010. The requirements for pre-treatment and the composition of the load of a desired load size out of the specified load items shall be applied.

The **base load** shall not be used for more than 80 **test runs**. Compliance with IEC 60456:2010, Annex J is not required.

Before each **test run** the **base load** shall be washed without detergent in order to remove detergent residues and shall undergo a heat treatment for 10 min in water at 90 °C and dried and stored under appropriate conditions to avoid re-contamination.

5.3.9 Measuring equipment for assessing temperature profile

The temperature profile shall be measured by temperature loggers placed among the **base load** items and following the load during the wash procedure. The temperature loggers shall comply with the specifications in Table 9.

Table 9 – Specifications for temperature logger

Temperature range	0 °C to 100 °C
Accuracy	≤ 0,5 °C over full range
Resolution	≤ 0,1 °C
Response time TC(10%-90%) water	≤ 2 min
Response time TC(10%-90%) moving air (2 m/s)	≤ 5 min
Sampling rate	≤ 10 s
Max. weight	70 g
NOTE TC(10%-90%) is the time for the sensor to traverse between 10% and 90% of its final value. Response time can also be expressed as TC 63% value. The 63% figure is the time for the sensor to reach 63% of its final value. TC(10%-90%) and TC 63% value are approximately of the same order of magnitude for a given sensor.	

5.3.10 Measuring equipment for water consumption

Equipment for measuring water consumption shall comply with the specifications of IEC 60456:2010, 5.5.

6 Tests

6.1 Test method principles

Bio monitors are used to determine microorganism reduction in domestic laundry processes and possible **cross contamination** of microorganisms.

Test conditions, materials, equipment and instrumentation shall be in accordance with IEC 60456, unless otherwise specified.

6.2 Preparation of test washing machine

Prior to each **test run** the **test washing machine** shall be conditioned with a 85 °C or 90 °C **programme**.

If this is not possible, the appliance shall be filled directly with no less than 50 l of water at a temperature not lower than 90 °C and two runs shall be performed.

Afterwards 2 rinsing cycles with cold water shall be made to cool down the appliance.

NOTE An adequate hygiene status of the machine can be monitored with appropriate methods, e.g. contact plates.

6.3 Preparation of test microorganisms and bio monitors

6.3.1 Cultures

The test microorganisms and their stock cultures shall be prepared and stored according to EN 12353.

6.3.1.1 Working cultures of *Candida albicans*

For preparation of working culture of *Candida albicans*, subculture from the stock culture by streaking onto at least two plates containing MEA shall be used.

After incubation of 42 h to 48 h at (30 ± 1) °C, a second subculture shall be prepared from the first subculture in the same way and incubated. The second subculture is used to prepare the **bio monitors**.

From this second subculture, a third subculture shall be produced in the same way. The third subculture shall be used for the verification analyses (see 6.5). No fourth subculture shall be prepared.

If it is not possible to prepare a second subculture on a particular day, a 72 h subculture can be used for subsequent subculturing, provided that the subculture has been kept in the incubator during the 72 h period. Under these circumstances, a further 48 h subculture shall be prepared before proceeding.

6.3.1.2 Yeast test suspensions (*Candida albicans*) according to EN 1650

The following procedure shall be followed.

a) Take 10 ml of diluting agent (see 5.2.2.3) and place in a 100 ml flask. Take the working culture (see 6.3.1.1) and transfer a loop of the cells into the diluting agent (see 5.2.2.3) by rubbing the loop against the wet wall of the flask to dislodge the cells before immersing in the diluting agent Shake the flask for 3 min using a shaking apparatus (see 5.3.6).

b) Adjust the number of cells in the suspension to $1,5 \times 10^8$ cfu/ml to $5,0 \times 10^8$ cfu/ml using the diluting agent, estimating the number of colony forming units (cfu) by any suitable means. Maintain this test suspension at 4 °C and use within 2 h.

NOTE The use of a spectrophotometer for adjusting the number of cells is highly recommended (about 620 nm wavelength – cuvette 10 mm path length). Each laboratory should therefore produce calibration data knowing that suitable values of optical density are generally found between 0,200 and 0,350. To achieve reproducible results of this measurement it may be necessary to dilute the test suspension, e.g. 1+9. A colorimeter is a suitable alternative.

c) For counting, prepare 10^{-6} and 10^{-7} dilutions of the test suspension using the diluting agent.

Take a sample of 1,0 ml of each dilution in duplicate and inoculate using the pour plate or the spread plate technique.

When using the pour plate technique, transfer each 1,0 ml sample into separate Petri dishes and add 15 ml to 20 ml melted MEA cooled to (45 ± 1) °C.

When using the spread plate technique, spread each 1,0 ml sample – divided into portions of approximately equal size – on an appropriate number (at least two) of surface dried plates containing MEA.

6.3.1.3 Working cultures of *P. putida* and *S. aureus*

For preparation of working cultures of *P. putida* and *S. aureus*, subcultures from the stock culture by streaking onto at least two plates containing TSA shall be used.

After 24 h incubation at (36 ± 1) °C, a second subculture shall be prepared from the first subculture in the same way and incubated. The second subculture is used to prepare the **bio monitors**.

From this second subculture, a third subculture shall be produced in the same way. The third subculture shall be used for the validation analyses (see 6.5). No fourth subculture shall be prepared.

If it is not possible to prepare a second subculture on a particular day, a 48 h subculture can be used for subsequent subculturing, provided that the subculture has been kept in the incubator during the 48 h period. Under these circumstances a further 24 h subculture shall be prepared before proceeding.

6.3.1.4 Bacterial test suspension (*P. putida* and *S. aureus*) according to EN 1276

The following procedure shall be followed.

a) Take 10 ml of diluting agent (see 5.2.2.3) and place in a 100 ml flask. Take the working culture (see 6.3.1.1.) and transfer a loop of the cells into the diluting agent by rubbing the loop against the wet wall of the flask to dislodge the cells before immersing in the diluting agent. Shake the flask for 3 min using an electromechanical agitator (see 5.3.6).

b) Adjust the number of cells in the suspension to $1,5 \times 10^9$ cfu/ml to $5,0 \times 10^9$ cfu/ml using the diluting agent (5.2.2.3), estimating the number of cfu by any suitable means. Maintain this test suspension at 4 °C and use within 2 h.

NOTE The use of a spectrophotometer for adjusting the number of cells can be recommended (about 620 nm wavelength – cuvette 10 mm path length). Each laboratory should therefore produce calibration data for each test organism knowing that suitable values of optical density are generally found between 0,150 and 0,460. To achieve reproducible results of this measurement it may be necessary to dilute the test suspension, e.g. 1+9. A colorimeter is a suitable alternative.

c) For counting, prepare 10^{-7} and 10^{-8} dilutions of the test suspension using the diluting agent.

Take a sample of 1,0 ml of each dilution in duplicate and inoculate using the pour plate or the spread plate technique.

When using the pour plate technique, transfer each 1 ml sample into separate Petri dishes and add 15 ml to 20 ml melted TSA, cooled to (45 ± 1) °C.

When using the spread plate technique, spread each 1,0 ml sample – divided into portions of approximately equal size – on an appropriate number (at least two) of surface dried plates containing TSA.

6.3.2 Bio monitors

6.3.2.1 Microorganism carriers

Microorganism carriers are described in 5.3.4.

6.3.2.2 Inoculation of the carriers

Five microorganism carriers shall be used per microorganism strain and **test run**.

The microorganism carriers are dipped in the test microorganism suspension and repeatedly turned by sterilized tweezers to wet them completely and remove all air bubbles.

The microorganism carriers shall rest for 20 min to 30 min in the suspension and are then transferred with a forceps in open Petri dishes for drying for a minimum of 3 h in an **incubator** at a temperature of (36 ± 1) °C.

NOTE In order to guarantee satisfactory drying after 3 h, it is necessary to ensure a minimal humidity in the incubator.

Each inoculated carrier is then transferred by sterilized tweezers into a separate bag. The 5 bio monitors of each test microorganism shall be placed in 5 cotton bags.

In addition, 2 inoculated carriers for each test microorganism shall be prepared to be used for validation (see 6.5).

Store the **bio monitors** at room temperature in a closed Petri dish and use within one week after drying.

6.4 Main test

6.4.1 General

The **programme** and size of the **base load** used for the main test shall be selected according to manufacturer's specification.

Split the dry **base load** into 2 similar parts. Fill one part into the **washing machine** drum, place the bio monitor bags (see 6.3.2.2) and the data logger (see 5.3.9) in the middle and finish loading with the second part of the **base load**. It is not necessary to follow the loading process according to IEC 60456.

Run the tested **programme** and analyse the **bio monitor** according to 6.4.2.

At least 3 valid **test runs** shall be performed for the tested **programme**.

6.4.2 Evidence of test microorganisms

To determine the amount of microorganisms on the bio monitors after the tested programme, each bio monitor is placed into 10 ml of neutralisation solution (see 5.2.2.4) and mixed vigorously with an electromechanical agitator (see 5.3.6) for 3 min before plating.

6.4.2.1 Microorganism plating

Dilution series shall be plated in duplicates.

For the preparation of the dilution series a sterile tip shall be used. Starting with the highest dilution, the tip should be rinsed three times with this solution. Then 0,1 ml are plated with a Drigalski spatula in rotary motion on an appropriate culture media plate. For each plate a sterile spatula shall be used.

Appropriate colony counts of each dilution shall be used for microorganism quantification (15 cfu to 300 cfu per plate).

For a further differentiation it is necessary to subcultivate some colonies on selective culture media as described in 5.2.2.

6.5 Validation

6.5.1 Enumeration of microorganisms before exposition N_0 (bio monitor reference)

For each **test run**, the microorganism content of 2 **bio monitors** per test microorganism strain shall be determined before exposition to the tested **programme**. The determination of microorganism counts on the carriers is performed as described in 6.4.2. The weighted average mean value ($\log_{10}$) of the 2 **bio monitors** is the basis for the calculation of reduction factors of the **bio monitor** after exposition (N_0, see 7.1) to the tested **programme**.

6.5.2 Negative control (cross contamination)

For the detection of **cross contamination**, 5 sterile microorganism carriers shall undergo the same process described in 6.4.1 for the **bio monitors**.

After running the tested **programme**, the microorganism count on the former sterile carriers is determined as described in 6.4.2. Appropriate culture media plates (see 5.2.2) for selective qualification of the test organisms shall be used for each microorganism carrier.

6.5.3 Determination of water quality

To ensure appropriate microbiological water quality, 1 ml of the water used for the washing process is examined on relevant culture media plates (5.2.2), respectively by plating or pouring and incubating it as described before.

6.5.4 Determination of water quantity in the main wash

The amount of water in the main wash for each test run of the tested programmes shall be determined as described in IEC 60456.

For a valid **test run**, the amount of water in the main wash during the main test shall not differ by more than ± 15 % from the water consumption determined according to 5.2.3.2

7 Evaluation

7.1 Log reduction

The reduction factors for each **test run** and microorganism strain are calculated as follows.

reduction factor per microorganism strain = $\log(N_0/N)$

where

N_0 is the average value of the microorganism content of the positive control (see 6.5.1);

N is the average of microorganism number of the 5 **bio monitors**, after exposition to the tested **programme**.

The standard deviation shall be calculated for at least 3 valid **test** runs for N_0, N and the reduction factors.

7.2 Cross contamination

Any growth of microorganisms after an incubation period of the control sterile swatches (see 6.5.2) shall be stated in cfu/sterile microorganism carrier.

If no growth could be detected this shall also be stated as < 100 cfu/sterile microorganism carrier.

8 Test report

The test report shall contain the following information:

- Date/period of testing
- Type of **washing machine** according to IEC 60456
- **Programme** tested
- Mass of **base load** in kg
- Ambient conditions: voltage of electricity supply, ambient temperature and humidity
- Used test microorganisms
- Drying time of **bio monitor**
- Microorganism number per microorganism type and **bio monitor**, before test
- Microorganism number per microorganism type and **bio monitor**, after test
- Reduction factor per microorganism type (including standard deviation)
- Microorganism transfer to sterile carriers

- water **temperature profile** during **test runs**
- Production data of **bio monitors** (for commercially available **bio monitors**: lot-number, production data and quality control date)

The test report may contain additional information e.g. determined according to IEC 60456. Examples are:

- Energy consumption
- Water consumption (total, main wash, rinsing)
- **Programme** time
- Water inlet temperature
- Age of **bio monitors**/drying time of **bio monitors** before using

Annex A
(informative)

Microorganism reduction in household washing machines with risk class 1 test microorganisms for internal development purpose

A.1 Scope

This alternative protocol allows the use of risk class 1 microorganisms for **washing machine** preevaluations and internal development tests.

For a final assessment of the microorganism reduction in a **washing machine**, the procedure described in this PAS shall be carried out.

A.2 General recommendation

Although the test is performed with risk class 1 microorganisms which are not pathogenic, the handling of the bio monitors should be carried out by trained staff with knowledge in sterile techniques. Working incautiously can affect or even bias the test results.

Even though small amounts of risk class 1 microorganisms can be spilled and discarded without restrictions, it is general good laboratory practice to autoclave agar plates and buffer solutions with microorganisms before discarding or discharging them.

The washing liquor can be discharged without any special disinfection treatments.

A.3 Material and reagents

A.3.1 Microorganisms on bio monitors

The following microorganisms, the first two bacteria strains and the last one a yeast strain, can be used:

- *Pseudomonas fluorescens* (ATCC 17397 / DSM 50091)
- *Staphylococcus arlettae* (ATCC 43957 / DSM 20672)
- *Saccharomyces cerevisiae* (ATCC 9763 / DSM 1333)

NOTE For indications about possible suppliers of ready-to-use **bio monitors**, see Annex B.

A.3.2 Water for culture media and solutions

As described in 5.2.2.2.

A.3.3 Culture media and solutions

A.3.3.1 Tryptone Soy Agar (TSA)

As decribed in 5.2.2.1.1.

A.3.3.2 Sabouraud Dextrose Agar

The composition of Sabouraud Dextrose Agar shall be according to Table A.1.

Table A.1 – Composition of Sabouraud Dextrose Agar

Description	Specification
Pancreatic digest of casein	5,0 g/l
Peptic digest of animal tissue	5,0 g/l
Dextrose monohydrate	40,0 g/l
Agar	12,0 g/l
Final pH	5,6 ± 0,2

A.3.3.3 Sabouraud Dextrose Agar with Chloramphenicol

As described in 5.2.2.1.2.

A.3.3.4 Cetrimide Agar

As described in 5.2.2.1.3.

A.3.3.5 Baird-Parker Agar

As described in 5.2.2.1.4.

A.3.3.6 Malt Extract Agar (MEA)

As described in 5.2.2.1.5.

A.3.3.7 Columbia CNA Agar

The composition of Columbia CNA Agar shall be according to Table A 2.

Table A.2 – Composition of Columbia CNA Agar

Description	Specification
Pancreatic digest of casein	5,0 g/l
Peptic digest of animal tissue	8,0 g/l
Yeast enriched peptone	10,0 g/l
Corn starch	1,0 g/l
Sodium chloride	5,0 g/l
Colistin	0,015 g/l
Nalidixic acid	0,01 g/l
Agar	14,0 g/l
Defibrinated sheep blood	5 %
Final pH	7,3 ± 0,2

A.3.3.8 Tryptone Soy Broth (TSB)

As described in 5.2.2.1.6.

A.3.3.9 Diluting agent

As described in 5.2.2.3.

A.3.3.10 Neutralisation solution

As described in 5.2.2.4.

A.3.4 Detergent

As described 5.2.3.

A.4 Equipment

A.4.1 Incubator

As described in 5.3.2.

A.4.2 Autoclave

As described in 5.3.3.

A.4.3 Microorganism carrier

The microorganism carriers are swatches made of standard cotton fabric in accordance with ISO 2267 with the dimension of 1,0 cm × 1,0 cm or Ø = 2,5 cm, boiled 3 times in distilled water, autoclaved, dried at room temperature and kept sterile prior to use, as described in 5.3.4.

A.4.4 Pipettes

As described in 5.3.5.

A.4.5 Electromechanical agitator

As described in 5.3.6.

A.4.6 Centrifuge

As described in 5.3.7.

A.4.7 Base load

As described in 5.3.8.

A.4.8 Measuring equipment for assessing temperature profile

As described in 5.3.9.

A.4.9 Measuring equipment for water consumption

As described in 5.3.10.

A.5 Tests

A.5.1 Test method principles

Bio monitors are used to determine microorganism reduction in domestic laundry processes and possible **cross contamination** of microorganisms.

Test conditions, materials, equipment and instrumentation shall be in accordance with IEC 60456, unless otherwise specified.

A.5.2 Preparation of washing machine

As described in 6.2.

A.5.3 Preparation of test organisms and bio monitors

Bio monitors can be prepared freshly or ready-to-use **bio monitors** can be used.

A.5.3.1 Cultures

The test microorganisms and their stock cultures have to be prepared and stored according to EN 12353.

A.5.3.1.1 Working culture for *Saccharomyces cerevisiae*

For preparation of working culture of *Saccharomyces cerevisiae* follow the same procedure described in 6.3.1.1 for *Candida albicans*.

A.5.3.1.2 Yeast test suspension according to EN 1650

Follow the same procedure described in 6.3.1.2 for *Candida albicans*.

A.5.3.1.3 Working culture for *P.fluorescens* and *S. arlettae*

For preparation of working culture of *P.fluorescens* and *S. arlettae*, follow the same procedure described in 6.3.1.3 for *P. putida* and *S. aureus*.

A.5.3.1.4 Bacterial test suspension

Follow the same procedure described in 6.3.1.4 for *P. putida* and *S. aureus*.

A.5.3.2 Bio monitors

A.5.3.2.1 Microorganism carriers

Microorganism carriers are described in A.4.3.

A.5.3.2.2 Inoculation of the carriers

Five microorganism carriers shall be used per microorganism strain and **test run**.

The microorganism carriers are dipped in the test microorganism suspension and repeatedly turned by sterilized tweezers to wet them completely and remove all air bubbles.

The microorganism carriers are kept for 20 min to 30 min in the suspension and then are transferred with a forceps in open Petri dishes for drying in an **incubator** for a minimum of 3 h at a temperature of (36 ± 1) °C for bacterial strains and at a temperature of (30 ± 1) °C for the yeast strain.

NOTE In order to guarantee satisfactory drying after 3 h, it is necessary to ensure a minimal humidity in the incubator.

Five inoculated carriers for each test microorganism are then transferred by sterilized tweezers in 5 separate cotton bags.

Store the **bio monitors** at room temperature in closed Petri dish and use within one week after drying.

If commercially available **bio monitors** are used, the **bio monitors** shall be stored in a refrigerator until use. Five commercially available **bio monitors** per test microorganism strain and **test run** shall be transferred by sterilized tweezers into 5 separate cotton bags.

In addition, 2 inoculated carriers for each test microorganism are used for validation (see A.5.5).

A.5.4 Main test

A.5.4.1 General

As described in 6.4.1.

A.5.4.2 Evidence of test microorganisms

To determine the amount of microorganisms on the **bio monitors** after the tested **programme**, each **bio monitor** is placed into 10 ml of neutralisation solution (see A.3.3.10) and mixed vigorously with an electromechanical agitator (see 5.3.6) for 1 min. The solution shall stand for 60 min at room temperature; afterwards, it is again mixed with an electromechanical agitator for 1 min before plating.

A.5.4.2.1 Plating microorganisms

For preparing the dilution series (Figure A.1), a sterile tip has to be used. 1 ml of the bio monitor suspension is transferred to another 9 ml of neutralisation solution (A.3.3.10). This first dilution is mixed and again 1 ml is transferred to a second dilution. This shall be done up to dilution v^{-5}. 0,1 ml of every dilution will be plated with a Drigalsky spatula in rotary motion on a Tryptone Soy Agar plate (A.3.3.1) for the bacterial bio monitors and on the Sabouraud Dextrose Agar (A.3.3.2) for the yeast bio monitors. For each plate a sterile spatula has to be used.

The plates shall be incubated at a temperature of (30 ± 1) °C for *Saccharomyces cerevisiae* or at a temperature of (36 ± 1) °C for *Staphylcoccus arlettae* and *Pseudomonas fluorescens* for 1 to 5 days.

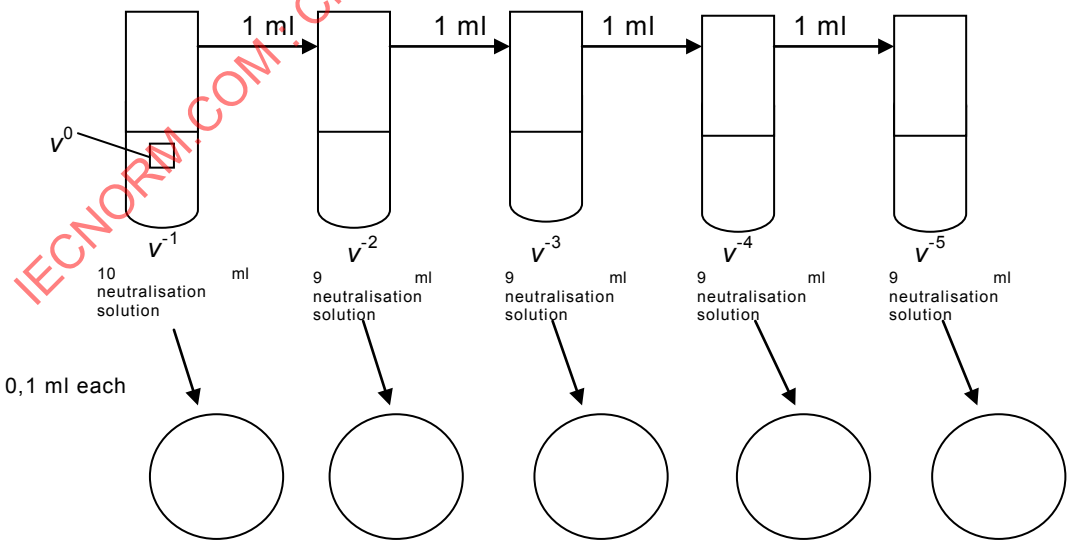

Figure A.1 – Scheme for preparing a dilution series

Appropriate colony counts of the dilutions shall be used for microorganism quantification. All culture media plates with colony counts between 15 and 300 cfu are used for further analysis; the other plates are discarded.